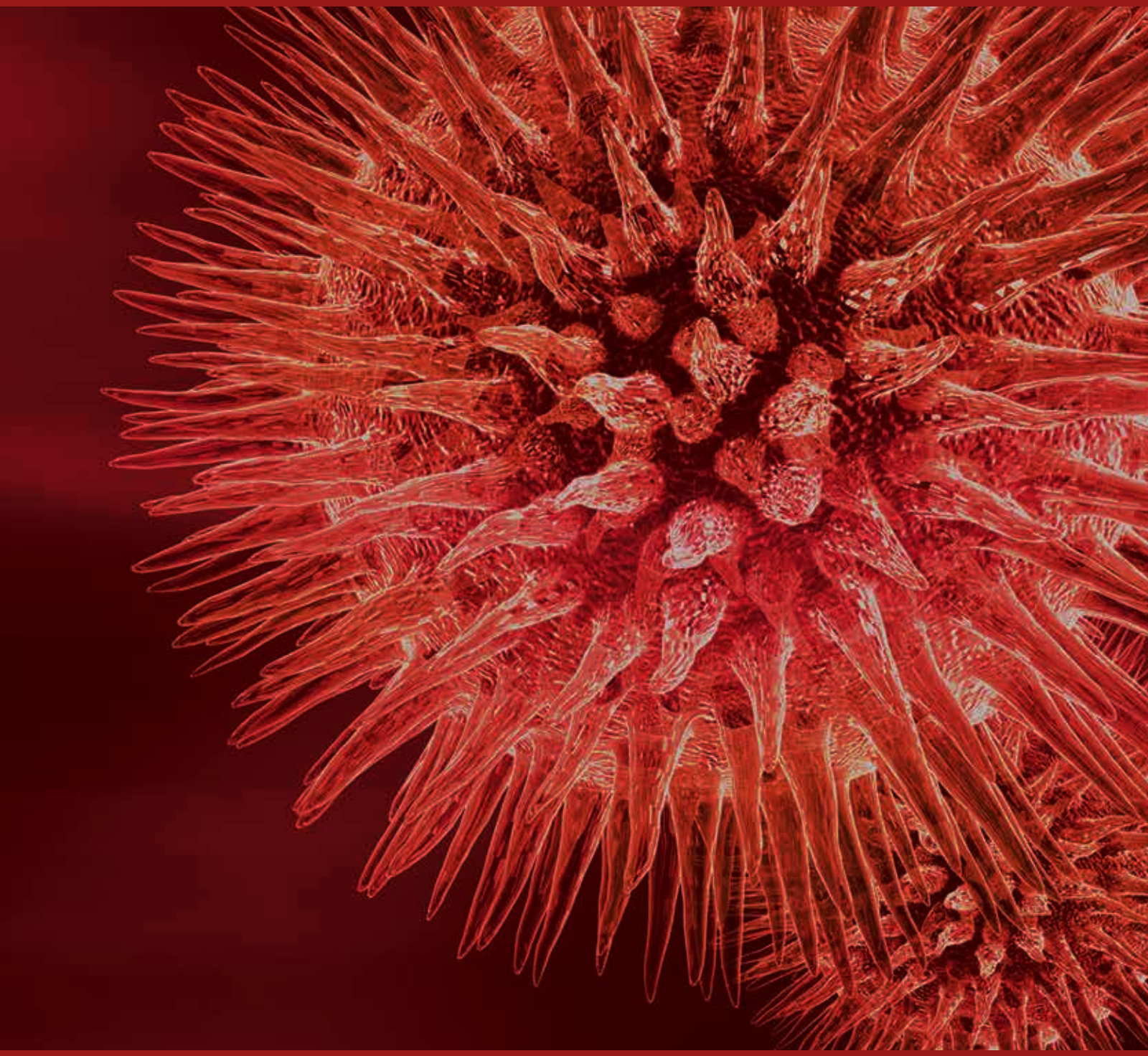
BioMed Research International

Endothelium in Diseased States

Guest Editors: Iveta Bernatova, Ramaroson Andriantsitohaina, Silvia M. Arribas, and Vladimir V. Matchkov



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Editorial
Endothelium in Diseased States

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Endothelium is the endocrine organ essential for maintenance of homeostasis in the entire body. Endothelium operates by a broad spectrum of signaling molecules controlling the contractile state of vascular smooth muscles and cardiomyocytes; long distance intercellular synchronization within the vascular wall; adhesive, coagulant, and rheological properties of blood; and permeability of the vascular wall. Dysfunctions in these signaling pathways result in loss of the important homeostatic functions as well as in engagement of the endothelium into activities leading to pathologies. This represents the two sides of the coin, where endothelium can be either a protective or "health-threatening" organ depending on the signaling involved. Endothelial dysfunction (ED) was observed in aging as well as in major lifestyle-related diseases suggesting that the endothelium can serve as a target for prevention and treatment of various diseases. Significant progress achieved in the identification of new endothelium-dependent signaling pathways and characterization of their role in various pathological states has been made since the identification of nitric oxide (NO) as an endothelium-derived relaxing factor. This special issue aims to highlight this progress and stimulate further this development. Variable functions of the endothelium are presented in six reviews, seven research articles, and two clinical studies.

The review of G. Favero et al. summarizes the endothelial function in major cardiovascular diseases and diabetes, endothelial alterations due to aging and smoking, and beneficial effects of physical activity and dietary products for endothelial function. Recent insights into the paracrine modulation of cardiomyocyte contractility by cardiac endothelial cells were reviewed by J. Noireaud and R. Andriantsitohaina, who suggested that some of the recently considered deleterious signals, like reactive oxygen species (ROS), need to be carefully reevaluated.

The review by I. Bernatova summarized a complex cross talk among the individual endothelium-derived factors and mentioned a possible classification of ED. In addition, this review analyzed the role of endothelium in the development of hypertension in various experimental models and it concluded that ED might be both a cause and the consequence of high blood pressure. Two other studies by A. Puzserova et al. and by P. Slezak et al. investigated the endothelial function in rats with genetic predispositions to hypertension, taking into account either aging in early periods of life or exposure to the chronic stress. A. Arnalich-Montiel et al. showed that short-term treatment of hypertensive rats with the β-blocker esmolol not only improved coronary artery remodeling but also normalized endothelium-dependent relaxation due to improved NO bioavailability and reduction of oxidative stress. S. Liskova et al. studied the influence of hypertension and aging on the norepinephrine-induced vasoconstriction mediated by Ca^{2+}-dependent Cl^{-} channels.

L. Pernomian et al. demonstrated the negative effect of ROS on *Mas* receptor-mediated signaling and carotid artery flow in diabetic rats. The review by A. Magenta et al. summarized the molecular mechanisms underpinning endothelial dysfunction in diabetic patients. The authors paid special attention to an important regulator of intracellular ROS production—p66Shc protein. V. Golubinskaya et al. studied the effect of myeloperoxidase deficiency on endothelial function in mice and found that myeloperoxidase deficiency failed to potentiate endothelium-dependent relaxation.

A balance between NO and ROS in rats exposed to intermittent and continuous chronic hypoxia was studied by P. Siques et al. The authors showed that continuous hypoxia produced larger nitrosative damage compared to intermittent exposure that could be related to the larger severity of cardiovascular alterations. The role of NO in cardiovascular and hypoxia-related respiratory diseases and the involvement of asymmetric dimethylarginine and its degrading enzyme dimethylarginine dimethylaminohydrolase in development of ED were reviewed by N. Lüneburg et al. S. H. van Ierssel et al. discussed the crucial role of endothelium in the development of organ failure and secondary ischemia. Their review pointed towards possible prognostic function of endothelial progenitor cells and endothelial microparticles as biomarkers for endothelial repair and damage, respectively.

Last but not least, two clinical studies completed this special issue. Study by L. M. Cotie et al. underlined the beneficial effect of exercise and diet on endothelial function in young obese woman. Another clinical study by K. D. Currie et al. examined acute endothelial responses to exercise in patients with coronary artery disease and found its correlation with the degree of resting endothelial dysfunction.

All articles involved in this special issue had brought about new and valuable information on the role of endothelium in health and diseases.

As the guest editors of this special issue we would like to acknowledge all authors who contributed either by reviewing recent literature or by original experimental and clinical studies, making this issue valuable for diverse audience of researchers interested in the endothelium in various diseased states.

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Research Article

Mas-Mediated Antioxidant Effects Restore the Functionality of Angiotensin Converting Enzyme 2-Angiotensin-(1–7)-*Mas* Axis in Diabetic Rat Carotid

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We hypothesized that endothelial AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species during type I-diabetes impairs carotid ACE2-angiotensin-(1–7)-*Mas* axis functionality, which accounts for the impaired carotid flow in diabetic rats. We also hypothesized that angiotensin-(1–7) chronic treatment of diabetic rats restores carotid ACE2-angiotensin-(1–7)-*Mas* axis functionality and carotid flow. Relaxant curves for angiotensin II or angiotensin-(1–7) were obtained in carotid from streptozotocin-induced diabetic rats. Superoxide or hydrogen peroxide levels were measured by flow cytometry in carotid endothelial cells. Carotid flow was also determined. We found that endothelial AT_1-activated NAD(P)H oxidase-driven generation of superoxide and hydrogen peroxide in diabetic rat carotid impairs ACE2-angiotensin-(1–7)-*Mas* axis functionality, which reduces carotid flow. In this mechanism, hydrogen peroxide derived from superoxide dismutation inhibits ACE2 activity in generating angiotensin-(1–7) seemingly by activating $I_{Cl,SWELL}$, while superoxide inhibits the nitrergic *Mas*-mediated vasorelaxation evoked by angiotensin-(1–7). Angiotensin-(1–7) treatment of diabetic rats restored carotid ACE2-angiotensin-(1–7)-*Mas* axis functionality by triggering a positive feedback played by endothelial *Mas* receptors, that blunts endothelial AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species. *Mas*-mediated antioxidant effects also restored diabetic rat carotid flow, pointing to the contribution of ACE2-angiotensin-(1–7)-*Mas* axis in maintaining carotid flow.

1. Introduction

Vascular dysfunction triggered by type I-diabetes has been extensively described as an important risk factor for the development of carotid atherosclerosis in the genesis of cerebrovascular diseases, such as stroke [1–4]. The major mechanisms underlying diabetic vascular dysfunction result from changes in the functionality of the main systems involved in the control of arterial tonus [5–9], such as renin-angiotensin system (RAS). Indeed, the upregulation of angiotensin converting enzyme- (ACE-) angiotensin II-AT_1 axis from vascular RAS seems to play a crucial role in the pathogenesis of diabetic vascular dysfunction and complications. Yousif et al. [5, 6] and Pernomian et al. [8, 9] showed that type I-diabetes enhances the vasocontractile response evoked by angiotensin II [5, 6, 8, 9], which damages vascular function and contributes with atherogenesis by affecting both the vascular tone and the progression of vascular inflammation [10]. Moreover, angiotensin converting enzyme (ACE) inhibitors or AT_1 antagonists attenuate carotid atherosclerosis during diabetes by improving vascular function [11–13].

Despite the aggressive effects assigned to ACE-angiotensin II-AT_1 axis on vascular function during diabetic conditions, there is another important axis from RAS, namely, ACE2-angiotensin-(1–7)-*Mas* axis, that triggers opposite effects to those produced by the former [14, 15]. In this alternative axis, ACE homologue (ACE2) hydrolyzes

angiotensin II into angiotensin-(1–7) [16, 17], which is the endogenous ligand of *Mas* receptors [18]. In vessels, such as rat carotid, the activation of *Mas* receptors evokes a nitrergic relaxation [19] that has been correlated with vasoprotective effects in diabetic conditions [20]. This perspective has pointed the vascular ACE2-angiotensin-(1–7)-*Mas* axis as a potential therapeutic target to attenuate diabetic endothelial dysfunction and the subsequent vascular complications. Nevertheless, the vasoprotective therapeutic efficacy of drugs aimed at the activation of ACE2-Angiotensin-(1–7)-*Mas* axis depends on the integrity of the functionality of this axis during the disease. To the best of our knowledge, there are no evidences concerning the consequences of type I-diabetes on the functionality of vascular ACE2-angiotensin-(1–7)-*Mas* axis. However, our previous findings suggest an indirect evidence concerning these consequences: in rat carotid, type I-diabetes shifts the Gaussian-like shape of angiotensin II-evoked contraction curve into a sigmoidal shape, due to endothelial AT_1-activated NAD(P)H oxidase-driven generation of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) [8]. In turn, the Gaussian-like shape of angiotensin II-induced contraction curve in rat carotid results from the relaxation triggered by micromolar concentrations of angiotensin II, which is mediated by *Mas* receptors [19] probably upon the hydrolysis of angiotensin II into angiotensin-(1–7). Thus, our previous findings [8] allow us to hypothesize that endothelial AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species during type I-diabetes impairs the functionality of ACE2-angiotensin-(1–7)-*Mas* axis in rat carotid by inhibiting both the hydrolysis of angiotensin II into angiotensin-(1–7) and the nitrergic signaling pathway underlying *Mas* receptors activation. Considering the vasoprotective effects assigned to vascular ACE2-angiotensin-(1–7)-*Mas* axis [20], we also hypothesize that the impairment of the functionality of this axis would contribute to damage carotid blood flow and resistance in type I-diabetic rats. Furthermore, since the activation of *Mas* receptors inhibits NAD(P)H oxidase-driven generation of reactive oxygen species in endothelial cells [21], we expect that the chronic treatment of type I-diabetic rats with angiotensin-(1–7) would restore the functionality of carotid ACE2-angiotensin-(1–7)-*Mas* axis and carotid blood flow and resistance by restoring both the local hydrolysis of angiotensin II into angiotensin-(1–7) and the nitrergic signaling pathway underlying *Mas* receptors activation.

2. Material and Methods

The experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. A prior approval was granted by the Animal Ethics Committee of the Faculty of Medicine from Ribeirão Preto (FMRP) from the University of São Paulo (USP) in Brazil (approval reference number: 007/2009). Male Wistar rats (*Rattus norvegicus*) were used in this study. These animals were kept under a 12 light/12 dark cycle (light from 06:00 to 18:00 h) and fed with regular chow and water *ad libitum*.

2.1. Experimental Design and Animal Groups. Type I-diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 55 mg/kg) dissolved in citrate buffer (0.09 mol/L, pH 4.5) (day 0) in eight-week-old male Wistar rats (350–400 g). The control group was composed by age-matched normoglycaemic rats that underwent to citrate buffer injection. Fasting glucose levels were determined from rat tail blood samples prior to and 48 h (day 2) after STZ or vehicle injection, by using a one-touch glucometer (LifeScan Inc., Milpitas, CA, USA). Diabetic rats presented glycaemia higher than 300 mg/dL (Table 1). Six weeks after STZ or vehicle injection (day 42), the animals underwent to body weight measurement (Table 2), then they were sacrificed, and the experiments were performed [8, 9]. In some protocols, STZ- or vehicle-treated rats (eight-weeks old) were chronically treated with intraperitoneal daily injections of the selective ACE2 inhibitor DX600 (5 μg/kg/day) or with daily intraperitoneal injections of the selective *Mas* receptors agonist angiotensin-(1–7) (576 μg/kg/day), combined or not with the selective *Mas* receptors antagonist A779 (1 mg/kg/day), for six weeks [22]. All these treatments started at the time of STZ or vehicle injection.

2.2. Ex Vivo Arterial Reactivity Studies. The functionality of vascular ACE2-angiotensin-(1–7)-*Mas* axis was studied by functional assays of angiotensin II or angiotensin-(1–7) cumulative concentration-response relaxant curves, obtained in *ex vivo* arterial reactivity studies in carotid rings from control or diabetic rats.

2.2.1. Carotid Rings Preparation. Rats were sacrificed by abdominal aortic exsanguination and common carotid arteries were isolated. Carotid rings (4 mm) were placed in 5.0 mL of Krebs-Henseleit bicarbonate buffer (composition in mmol/L: NaCl 118.4; KCl 4.7; $CaCl_2$ 1.9; KH_2PO_4 1.2; $MgSO_4 \cdot 7H_2O$ 1.2; $NaHCO_3$ 25; $C_6H_{12}O_6$ 11.6) in organ bath chambers, gassed with 95% O_2 and 5% CO_2 and maintained at 37°C and pH 7.4. The rings were connected to an isometric force transducer (Letica Scientific Instruments, Barcelona, Spain) to measure changes in the isometric tension [8, 9, 23, 24]. After 60 min of stabilization at a resting tension of 1 g, carotid rings viability was tested with the appropriate molar concentration of phenylephrine that produces 50% of the maximum contraction response (EC_{50}) in each experimental group (phenylephrine EC_{50} = 0.1 μmol/L for control rat carotid or phenylephrine EC_{50} = 10 nmol/L for diabetic rat carotid), as previously determined by cumulative concentration-response curves for phenylephrine. The endothelial integrity was verified with the appropriate molar concentration of acetylcholine that produces 100% of the maximum relaxation response (EC_{100}) in each experimental group (acetylcholine EC_{100} = 1.0 μmol/L for control rat carotid or acetylcholine EC_{100} = 100 μmol/L for diabetic rat carotid), over phenylephrine-induced precontraction [8, 9]. For studies with endothelium-intact vessels, the ring was discarded if the maximum relaxation induced by acetylcholine did not reach 80–100%. When necessary, the endothelium was mechanically removed by gentle rubbing the vessel with

Table 1: Fasting blood glucose levels from control or diabetic rats.

Rats	Glycaemia ($mg \cdot dL^{-1}$)	
	Day 0	Day 2
Nontreated Control	68.7 ± 3.11	73.0 ± 6.35
Nontreated Diabetic	75.4 ± 5.17	392.6 ± 11.47*,#
DX600-treated control	70.5 ± 4.20	71.8 ± 5.23
DX600-treated diabetic	69.9 ± 6.31	402.7 ± 15.25*,#
Angiotensin-(1–7)-treated control	73.1 ± 5.19	67.9 ± 5.24
Angiotensin-(1–7)-treated diabetic	71.2 ± 4.98	385.9 ± 13.26*,#
A779-treated control	72.1 ± 4.57	76.3 ± 7.93
A779-treated diabetic	74.2 ± 7.21	406.0 ± 14.69*,#
Angiotensin-(1–7) + A779-treated control	67.5 ± 6.52	69.3 ± 6.45
Angiotensin-(1–7) + A779-treated diabetic	71.2 ± 7.09	409.1 ± 17.33*,#

The values are significantly different ($P < 0.01$; $n = 11$) from the respective control rats at the same day (∗) or the same rats at day 0 (#).

Table 2: Body weight from control or diabetic rats.

Rats	Body weight (g)	
	Day 0	Day 42
Nontreated control	374.9 ± 7.5	714.1 ± 10.2#
Nontreated diabetic	365.2 ± 6.9	286.3 ± 9.1*,#
DX600-treated control	361.5 ± 5.11	721.2 ± 11.4#
DX600-treated diabetic	378.0 ± 4.9	267.5 ± 10.3*,#
Angiotensin-(1–7)-treated control	373.8 ± 6.2	733.9 ± 8.7#
Angiotensin-(1–7)-treated diabetic	354.2 ± 8.2	275.3 ± 11.6*,#
A779-treated control	369.1 ± 5.7	729.4 ± 7.5#
A779-treated diabetic	372.4 ± 9.1	281.6 ± 13.2*,#
Angiotensin-(1–7) + A779-treated control	368.4 ± 7.2	719.1 ± 9.5#
Angiotensin-(1–7) + A779-treated diabetic	355.7 ± 6.9	272.3 ± 11.4*,#

The values are significantly different ($P < 0.01$; $n = 11$) from the respective control rats at the same day (∗) or the same rats at day 0 (#).

a thin wire. Endothelium was deemed absent when the relaxation response to acetylcholine did not occur [8, 9, 23, 24].

2.2.2. Experimental Protocols

(1) Cumulative Concentration Response Relaxant Curves for Angiotensin II. The functionality of vascular ACE2 (i.e., the activity of vascular ACE2 in generating angiotensin-(1–7)) was studied by relaxant curves induced by angiotensin II in rat carotid. This approach was supported by two evidences: (1) the angiotensin 1–7 *Mas* receptor antagonist, A779, partially inhibits the relaxation evoked by angiotensin II in rat carotid [19], suggesting that angiotensin II is converted into angiotensin-(1–7) in this bed; and (2) the unique enzyme that is able to convert angiotensin II into angiotensin-(1–7) in vascular tissues is ACE2 [17, 25].

Relaxation cumulative concentration-response curves for angiotensin II (10^{-9}–10^{-4} mol/L) were obtained in endothelium-intact or endothelium-denuded carotid rings from control or diabetic rats, over the precontraction induced by the appropriate molar concentration of phenylephrine that produces 80% of the maximum contraction response (EC_{80}) in each experimental group (phenylephrine EC_{80} = 1.0 μmol/L for control rat carotid or phenylephrine EC_{80} = 0.1 μmol/L for diabetic rat carotid). In this study, we have chosen phenylephrine EC_{80} instead of EC_{50} due to the higher magnitude of the precontraction evoked by the former concentration (too much closer to the maximum contraction). This avoids significant changes on the precontraction value upon pharmacological intervention. In functional assays, this is an important approach, since it avoids significant interferences from the precontraction magnitude variation on the relaxant response magnitude. Finally, EC_{80} can be safely used to induce a precontraction because it is as accurate for the homogeneous distribution of date as the EC_{50}, since both of them can be found at the central linear region of sigmoidal curves. The relaxant curves for angiotensin II were obtained in carotid rings from nontreated control or diabetic rats, in the absence or presence of DX600 (10 μmol/L, 30 min) [26], A779 (5.0 μmol/L, 30 min) [19] or the selective AT_2 antagonist, PD123,319 (0.5 μmol/L, 30 min) [19]. As posteriorly described in the Results section, this protocol confirms that ACE2 converts angiotensin II into angiotensin-(1–7), which in turn partially mediates the relaxation evoked by angiotensin II by activating *Mas* receptors. Thus, this protocol validates the functional studies of angiotensin II-evoked relaxant curves in rat carotid as a method to study vascular ACE2 functionality.

To verify if the reactive oxygen species derived from AT_1-activated NAD(P)H oxidase impair the functionality of ACE2 in diabetic rat carotid, angiotensin II-induced relaxant curves were obtained in the absence or presence of the selective AT_1 antagonist, losartan (1.0 μmol/L, 30 min) [27], the selective NAD(P)H oxidase inhibitor, apocynin (0.1 mmol/L, 30 min) [8, 23], the selective O_2^- scavenger, tiron (0.1 mmol/L, 30 min) [8, 23], or the selective H_2O_2 scavenger, PEG-catalase (250 U/mL, 30 min) [8, 23]. Also, we investigated the hypothesis that the reactive oxygen species-mediated activation of volume-sensitive Cl^- current ($I_{Cl,SWELL}$) impairs ACE2 functionality in diabetic rat carotid, by obtaining angiotensin II-evoked relaxant curves in the presence of the selective inhibitor of $I_{Cl,SWELL}$, DCPIB (10 μmol/L, 10 min) [28, 29]. This hypothesis was suggested based on the following evidences: (1) the cleavage of angiotensin II by ACE2 is reduced by increasing the physiological extracellular levels of Cl^- [30]; and (2) H_2O_2 generated from NAD(P)H oxidase-derived O_2^- can activate $I_{Cl,SWELL}$ in vascular smooth muscle cells, leading to the efflux of Cl^- and thus increasing Cl^- extracellular levels [31, 32].

In order to verify if the *Mas*-mediated effects against AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species restore the functionality of ACE2 in diabetic rat carotid, the relaxation curves for angiotensin II were obtained in carotid rings from control or diabetic rats that were chronically treated with angiotensin-(1–7). This protocol was not repeated in carotid rings from rats treated with angiotensin-(1–7) combined with A779 because angiotensin II-evoked relaxation is partially mediated by *Mas* receptors [19].

(2) Cumulative Concentration-Response Relaxant Curves for Angiotensin-(1–7). The functionality of vascular *Mas* receptors (i.e., nitrergic vasorelaxant response evoked by angiotensin-(1–7) upon *Mas* receptors activation) was studied by relaxation cumulative concentration-response curves for angiotensin-(1–7) (10^{-11}–10^{-5} mol/L), that were obtained in endothelium-intact or endothelium-denuded carotid rings from control or diabetic rats. In these protocols, carotid rings were also precontracted with phenylephrine EC_{80} (1.0 μmol/L for control rat carotid or 0.1 μmol/L for diabetic rat carotid), due to the reasons previously described.

The mediators of angiotensin-(1–7)-induced relaxation were characterized by obtaining these curves in carotid rings from nontreated control or diabetic rats, in the absence or presence of A779 (5.0 μmol/L, 30 min), PD123,319 (0.5 μmol/L, 30 min), the selective nitric oxide (NO) scavenger hydroxocobalamin (0.1 mmol/L, 30 min) [33], the nonselective NOS inhibitor L-NNA (0.1 mmol/L, 30 min), the selective neuronal NOS (*n*NOS) inhibitor L-NPA (50 nmol/L, 30 min), or the selective inducible NOS (*i*NOS) inhibitor 1400 W (10 nmol/L, 30 min) [9].

To verify if the reactive oxygen species derived from AT_1-activated NAD(P)H oxidase impair the functionality of angiotensin-(1–7)-*Mas* receptors in diabetic rat carotid, angiotensin-(1–7)-induced relaxant curves were obtained in the absence or presence of losartan (1.0 μmol/L, 30 min), apocynin (0.1 mmol/L), tiron (0.1 mmol/L), or PEG-catalase (250 U/mL). We also investigated if the reactive oxygen species-activated $I_{Cl,SWELL}$ impairs the functionality of angiotensin-(1–7)-*Mas* receptors in diabetic rat carotid, by obtaining angiotensin II-evoked relaxant curves in the presence of DCPIB (10 μmol/L, 10 min).

In order to verify if the *Mas*-mediated effects against AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species restore the functionality of angiotensin-(1–7)-*Mas* receptors in diabetic rat carotid, the relaxation curves for angiotensin II were obtained in carotid rings from control or diabetic rats that were chronically treated with angiotensin-(1–7). This protocol was not repeated in carotid rings from rats treated with angiotensin-(1–7) combined with A779 because angiotensin-(1–7)-evoked relaxation is mediated by *Mas* receptors [19].

2.3. Flow Cytometry in Endothelial Cells. To confirm the functional evidences that the *Mas*-mediated effects induced by the chronic treatment with angiotensin-(1–7) on ACE2-angiotensin-(1–7)-*Mas* axis functionality in diabetic rat carotid involve antioxidant actions against endothelial AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species (O_2^- and H_2O_2), flow cytometry assays were performed in carotid-derived endothelial cells, loaded with selective probes for reactive oxygen species.

Carotid arteries were isolated after abdominal aortic exsanguination. Thus, the arteries were longitudinally sectioned and endothelial cells were mechanically isolated by gentle friction with a plastic stem in plates containing Hanks' solution (composition in mmol/L: $CaCl_2$ 1.6; $MgSO_4$ 1.0; NaCl 145.0; KCl 5.0; NaH_2PO_4 0.5; dextrose 10.0; HEPES 10.0) at pH 7.4. The cell suspensions were centrifuged at 1.375 hg for 5 min, and the pellets were resuspended in 0.5 mL of Hanks's solution in a humidified incubator at 37°C until use [8, 9, 23, 24, 34]. Each *n* comprised a pool of six carotid arteries. The cell viability was previously determined by trypan blue staining (2%) and counting in a Neubauer chamber (Weber Scientific International, Germany).

The endothelial cells were loaded with the nonselective fluorescent dye for reactive oxygen species, dihydroethidium (DHE, 2.5 μmol/L, 20 min, 37°C) [8, 23] or with the selective fluorescent dye for H_2O_2, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCF-DA, 1 μmol/L, 20 min, 25°C) [9, 35].

The basal levels of O_2^- from endothelial cells isolated from carotid arteries of nontreated control or diabetic rats were measured in the absence or presence of tiron (0.1 mmol/L, 30 min), apocynin (0.1 mmol/L, 30 min), losartan (1.0 μmol/L, 30 min), or DCPIB (10 μmol/L, 10 min), added to the cellular samples during DHE loading. The basal levels of O_2^- were also measured in endothelial cells from carotid arteries of control or diabetic rats chronically treated with angiotensin-(1–7), combined or not with A779.

The basal levels of H_2O_2 from endothelial cells isolated from carotid arteries of nontreated control or diabetic rats were measured in the absence or presence of PEG-catalase (3,000 U/mL, 30 min) [36], tiron (0.1 mmol/L, 30 min), apocynin (0.1 mmol/L, 30 min), losartan (1.0 μmol/L, 30 min), or DCPIB (10 μmol/L, 10 min), added to the cellular samples

during CDCF-DA loading. The basal levels of H_2O_2 were also measured in endothelial cells from carotid arteries of control or diabetic rats chronically treated with angiotensin-(1–7), combined or not with A779.

2.4. Immunohistochemical Assays. The expression of ACE2 and *Mas* receptors, as well as the levels of angiotensin-(1–7), was assessed by immunohistochemical assays. These data were correlated with the functionality of ACE2-angiotensin-(1–7)-*Mas* axis in nontreated control or diabetic rat carotid arteries. The effects of the chronic treatment with angiotensin-(1–7) on ACE2 and *Mas* receptors expression in control or diabetic rat carotid were also studied. In turn, the expression of angiotensin-(1–7) was not assessed in carotid arteries from control or diabetic rats that were chronically treated with angiotensin-(1–7), since this treatment tends to increase the vascular levels of the heptapeptide. Furthermore, to confirm the functional evidences concerning the generation of angiotensin-(1–7) by ACE2 in rat carotid and the impairment of ACE2 activity in generating angiotensin-(1–7) in diabetic rat carotid, angiotensin-(1–7) levels were also assessed in carotid arteries from nontreated or DX600-treated control or diabetic rats.

As we aimed to evaluate the expression of ACE2, angiotensin-(1–7) and *Mas* receptors in each vascular layer, mainly in the endothelium, we have chosen immunohistochemistry instead of western blotting (WB) assays, since it is not viable to isolate endothelial cells from rat carotid to perform WB (each *n* of endothelial cells sample would comprise more than 20 carotid arteries to yield a sufficient protein level). In addition, angiotensin-(1–7) levels could not be evaluated by western blotting assays since the molecular weight of angiotensin-(1–7) is lesser than 2.0 kDa, what precludes the use of the available polyacrylamide or tricine gels and molecular weight markers.

After abdominal aortic exsanguination, carotid arteries were isolated and included in paraffin. Ion paraffin-included carotid rings were cut into 3 μm sections and mounted on poly-L-lysine-coated slides, which were rinsed with phosphate-buffered saline (PBS) and immersed in 3% H_2O_2 for 20 min to block endogenous peroxidase. Nonspecific protein binding was blocked with normal serum for 30 min [8, 9]. The sections were incubated with the primary antibody against ACE2 (1 : 100) [37], *Mas* receptors (1 : 250) [38], or angiotensin-(1–7) (1 : 100) [39] for 2 h at 25°C. Following washes in PBS, biotinylated pan-specific universal secondary antibody (1 : 300) was applied for 30 min. The slides were incubated with avidin-biotin peroxidase complex for 30 min. They were then counterstained by haematoxylin, dehydrated, and mounted with Permount. As negative controls, all specimens were incubated with an isotope-matched control antibody under identical conditions. The immunolabeling was considered positive when distinct red nuclear or cytoplasmic staining was homogenously present [8, 9].

2.5. Carotid Blood Flow Measurement. In order to verify if the functionality of local ACE2-angiotensin-(1–7)-*Mas* axis contributes to the control of carotid blood flow and resistance and if the *Mas*-mediated effects restore carotid blood flow and resistance in diabetic rats, these experiments were performed in control or diabetic rats that were chronically treated or not with DX600, A779 or with angiotensin-(1–7), combined or not with A779.

Rats were anaesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg), by intraperitoneal injection. The left and right common carotid arteries were exposed. A noninvasive transit-time flow probe (model 1.5RB; Transonic Systems, Inc., Ithaca, NY, USA) was placed around each carotid artery and connected to a flow meter (model T-206; Transonic system, USA). Basal mean blood pressure was determined in anaesthetized rats after femoral artery cannulation [9, 40]. Blood pressure and carotid flow were simultaneously measured after the slow infusion of normal saline (0.9%, 1 mL), that was used as volume expander, in order to prevent the eventual interferences related to the reduced tissue perfusion volume in diabetic rats, whose body weight is reduced (Table 2).

2.6. Data Analysis. Data were expressed as the mean ± S.E.M., and the differences between the mean values were assessed by one-way ANOVA and Bonferroni *post-hoc*. The significance level considered was 0.05. We chose one-way ANOVA as the statistical test since there was only one independent variable in the experimental design (i.e., the *treatment* factor), which was divided into two categorical (nominal) variables: (1) the *in vivo* treatment of rats with STZ, citrate buffer, DX600, angiotensin-(1–7), and/or A779; and (2) the *in vitro* pretreatment of carotid rings or carotid endothelial cells with DX600, A779, PD123,319, losartan, hydroxocobalamin, apocynin, tiron, PEG-catalase, or DCPIB. Endothelial removal is also considered as an *in vitro* pretreatment variable, since the removal of endothelial modulation on vascular responses can be equally reached by mechanical or chemical induction.

In the functional studies, relaxant responses were recorded as reductions in the muscular tone evoked by the preconstrictor agent and expressed as grams of tension (absolute relaxation values) on negative scale. The concentration-response curves were fitted using a nonlinear interactive fitting program (GraphPad Prism 5.00; GraphPad Software Inc., San Diego, CA). The maximum effect (E_{max}) of AngII or Ang-(1–7) was obtained from the nonlinear regression of the agonist curve.

In the flow cytometry analysis, the median values of the fluorescence intensity (FI) emitted by endothelial cells were determined using DIVA software and expressed in fluorescence units (U).

The percentage of the stained area was determined by ImageJ Program (1.46r, Wayne Rasband, NIH, USA) in immunohistochemical assays, in which it was delimited an area of 900 μm^2 for the media or an area of 45 μm^2 for the endothelium from carotid sections.

In the *in vivo* experiments, the mean carotid blood flow (F) was recorded by 10 min in a computational acquisition system (Dataq, USA), which provided an actual volume flow measurement in a resolution of 0.05 mL/min. Mean blood pressure (BP) baseline values were calculated as the average of

the 10 min recording, by using the acquisition system (Dataq, USA). F and BP were used to calculate the carotid resistance (R) by applying the formulae $R = BP/F$.

2.7. Drugs, Chemical Reagents, and Other Materials. They are STZ, angiotensin II, angiotensin-(1–7), losartan, PD123,319, hydroxocobalamin, L-NNA, 1400 W, apocynin, tiron, PEG-catalase, CDCF-DA, and DCPIB (Sigma, St. Louis, Mo., USA); DX600 (Anaspec Inc., Fremont, CA, USA); A779 (Bachem California Inc., Torrance, CA, USA); L-NPA (Tocris, Avonmouth, UK); DHE (Invitrogen, Carlsbad, CA, USA); ketamine (União Química, Jabaquara, SP, Brazil); xylazine (Calier Laboratory, Jubatuba, MG, Brazil); rabbit polyclonal anti-ACE2 antibody (Abcam, Cambridge, MA, USA); rabbit anti-angiotensin-(1–7) antibody (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA); rabbit polyclonal anti-angiotensin-(1–7)-*Mas* receptor antibody (Alomone Labs, Jerusalem, Israel); biotinylated universal secondary antibody, avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Universal, Vector Laboratories Inc. U.S. Headquarters, Burlingame, CA, USA).

3. Results

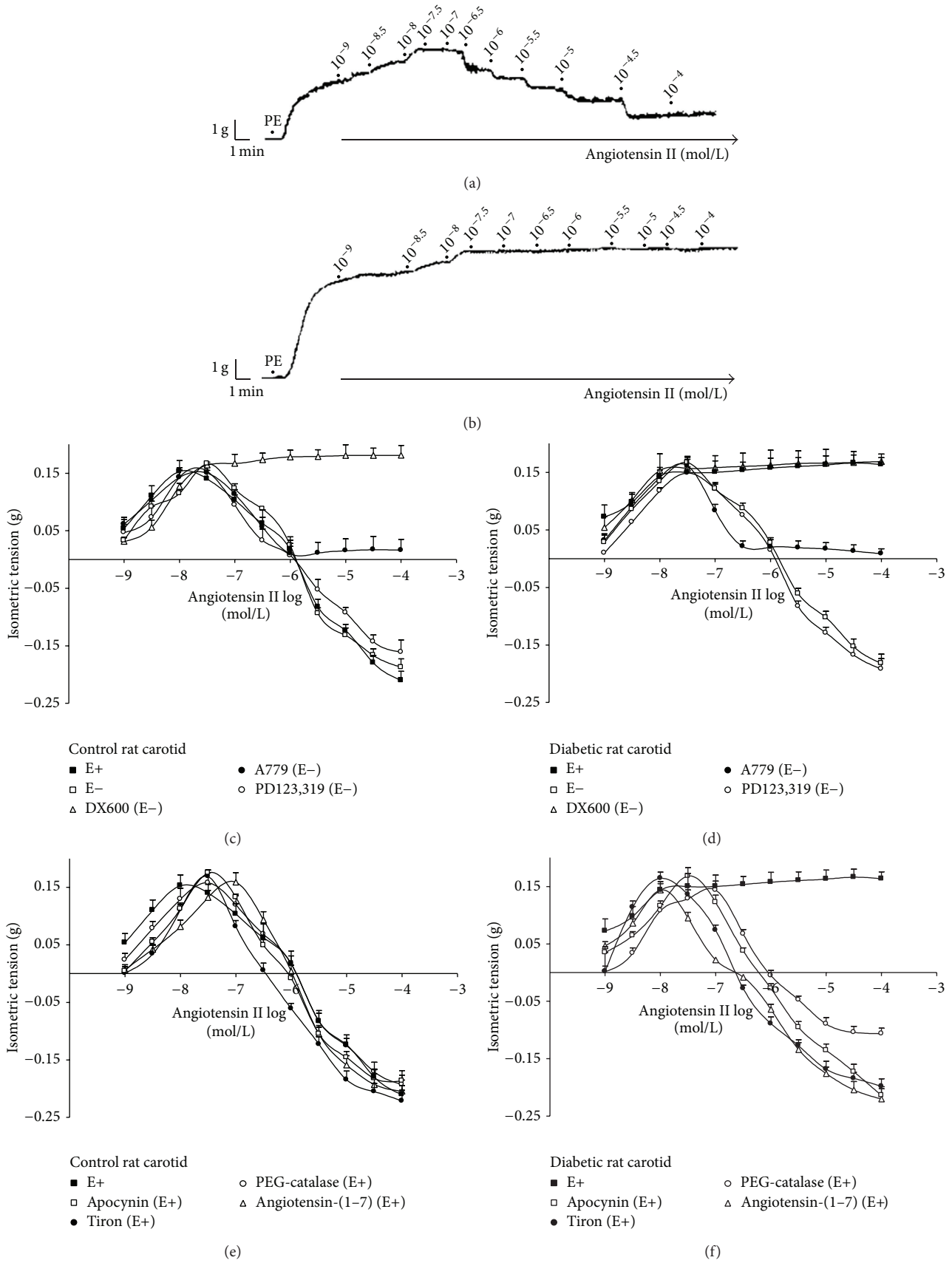
3.1. Angiotensin II-Induced Relaxation. Phenylephrine EC_{80} evoked a precontraction of 0.29 ± 0.021 g ($n = 9$) in endothelium-intact carotid rings from nontreated control rats. In these vessels, angiotensin II produced a biphasic response over phenylephrine-induced precontraction, which was characterized by a residual contraction (0.15 ± 0.019 g, $n = 9$) evoked by nanomolar concentrations of angiotensin II, followed by a relaxant response ($E_{max} = -0.36 \pm 0.028$ g, $n = 9$) induced by micromolar concentrations of angiotensin II (Figures 1(a) and 1(c)). Endothelium removal did not alter the magnitude of phenylephrine-induced precontraction (0.34 ± 0.032 g, $n = 9$) or the relaxant response evoked by angiotensin II ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*). In endothelium-denuded carotid rings from nontreated control rat, this relaxant response was completely inhibited by DX600 but partially inhibited by A779 (Figures 1(c) and 1(g)). Losartan inhibited the residual contraction evoked by angiotensin II and thus reduced the maximum relaxation triggered by angiotensin II in endothelium-intact carotid rings from nontreated control rat (Figures 3(a) and 3(c)). The magnitude of phenylephrine-induced precontraction in control rat carotid rings was not altered by DX600 (0.35 ± 0.031 g, $n = 9$), A779 (0.32 ± 0.025 g, $n = 9$), or losartan (0.29 ± 0.023 g, $n = 9$) ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*).

Phenylephrine EC_{80} evoked a precontraction of 0.51 ± 0.033 g ($n = 9$) in endothelium-intact carotid rings from nontreated diabetic rats. This value was significantly higher than that one evoked in endothelium-intact carotid rings from nontreated control rats ($P < 0.001$, *one-way ANOVA, Bonferroni post-hoc*). In carotid artery from nontreated diabetic rat, nanomolar concentrations of angiotensin II evoked a persistent residual contraction (0.17 ± 0.014 g, $n = 9$) over phenylephrine-induced precontraction (Figures 1(b) and 1(d)). Diabetes abrogated the relaxant response induced by micromolar concentrations of angiotensin II in rat carotid, which was completely restored by endothelium removal (this result required that the characterization of the mediators of angiotensin II-induced relaxation was performed in endothelium-denuded carotid rings from diabetic rats, as well as from control rats, to compare the responses). In endothelium-denuded carotid rings from nontreated diabetic rats, the relaxant response evoked by angiotensin II was completely inhibited by DX600 but partially inhibited by A779 (Figures 1(d) and 1(g)). Losartan inhibited the residual contraction induced by angiotensin II but allowed micromolar concentrations of angiotensin II to elicit a relaxant response in endothelium-intact carotid rings from nontreated diabetic rat (Figures 3(a) and 3(c)). The magnitude of phenylephrine-induced precontraction in carotid rings from nontreated diabetic rats was not altered by endothelium removal (0.46 ± 0.041 g, $n = 9$) or the pretreatment with DX600 (0.49 ± 0.037 g, $n = 9$), A779 (0.47 ± 0.028 g, $n = 9$), or losartan (0.52 ± 0.045 g, $n = 9$), when compared to nonpretreated endothelium-intact diabetic rat carotid ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*).

In endothelium-denuded carotid rings from nontreated control or diabetic rats, the maximum relaxation induced by angiotensin II in the presence of A779 had the same extent (in absolute values of g of tension) as the maximum contraction evoked by angiotensin II in these arteries (Figures 1(c), 1(d), and 1(g)).

Although the relaxation induced by angiotensin II has not been altered by apocynin, tiron, PEG-catalase, and DCPIB or by the chronic treatment with angiotensin-(1–7) in endothelium-intact carotid rings from nontreated control rats, this response was completely restored by apocynin or tiron and partially restored by PEG-catalase or DCPIB in endothelium-intact carotid rings from nontreated diabetic rats. Similar to the effects of apocynin or tiron, the chronic treatment with angiotensin-(1–7) completely restored the relaxation induced by angiotensin II in endothelium-intact carotid rings from diabetic rats (Figures 1(e), 1(f), 1(h), 3(a), and 3(c)). In control rat carotid, the magnitude of phenylephrine-induced precontraction was not altered by apocinin (0.28 ± 0.019 g, $n = 9$), tiron (0.27 ± 0.022 g, $n = 9$), PEG-catalase (0.30 ± 0.023 g, $n = 9$), angiotensin-(1–7) (0.32 ± 0.031 g, $n = 9$), or DCPIB (0.31 ± 0.024 g, $n = 9$). Also, in diabetic rat carotid, phenylephrine-induced precontraction was not altered by apocinin (0.53 ± 0.039 g, $n = 9$), tiron (0.49 ± 0.035 g, $n = 9$), PEG-catalase (0.50 ± 0.049 g, $n = 9$), angiotensin-(1–7) (0.51 ± 0.042 g, $n = 9$), or DCPIB (0.50 ± 0.033 g, $n = 9$), when compared to nonpretreated diabetic rat carotid ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*).

3.2. Angiotensin-(1–7)-Induced Relaxation. In carotid artery from nontreated control rat, angiotensin-(1–7) evoked a relaxant response ($E_{max} = -0.20 \pm 0.016$ g, $n = 9$) over the precontraction evoked by phenylephrine (0.30 ± 0.025 g, $n = 9$). This relaxant response was not altered by endothelium removal. In endothelium-denuded carotid rings from nontreated control rats, this relaxant response was



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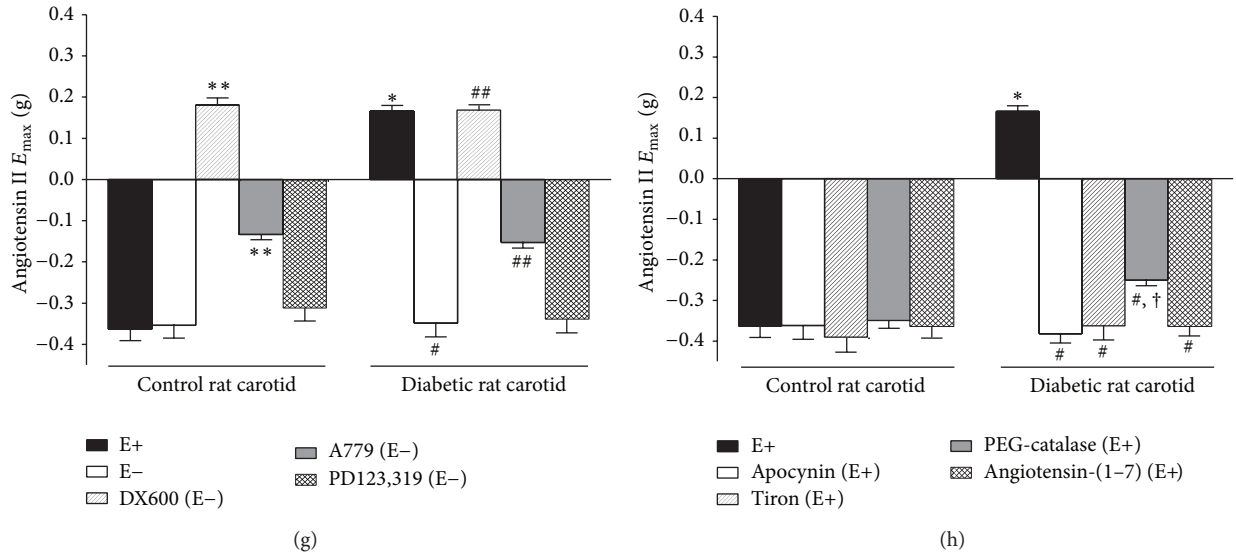


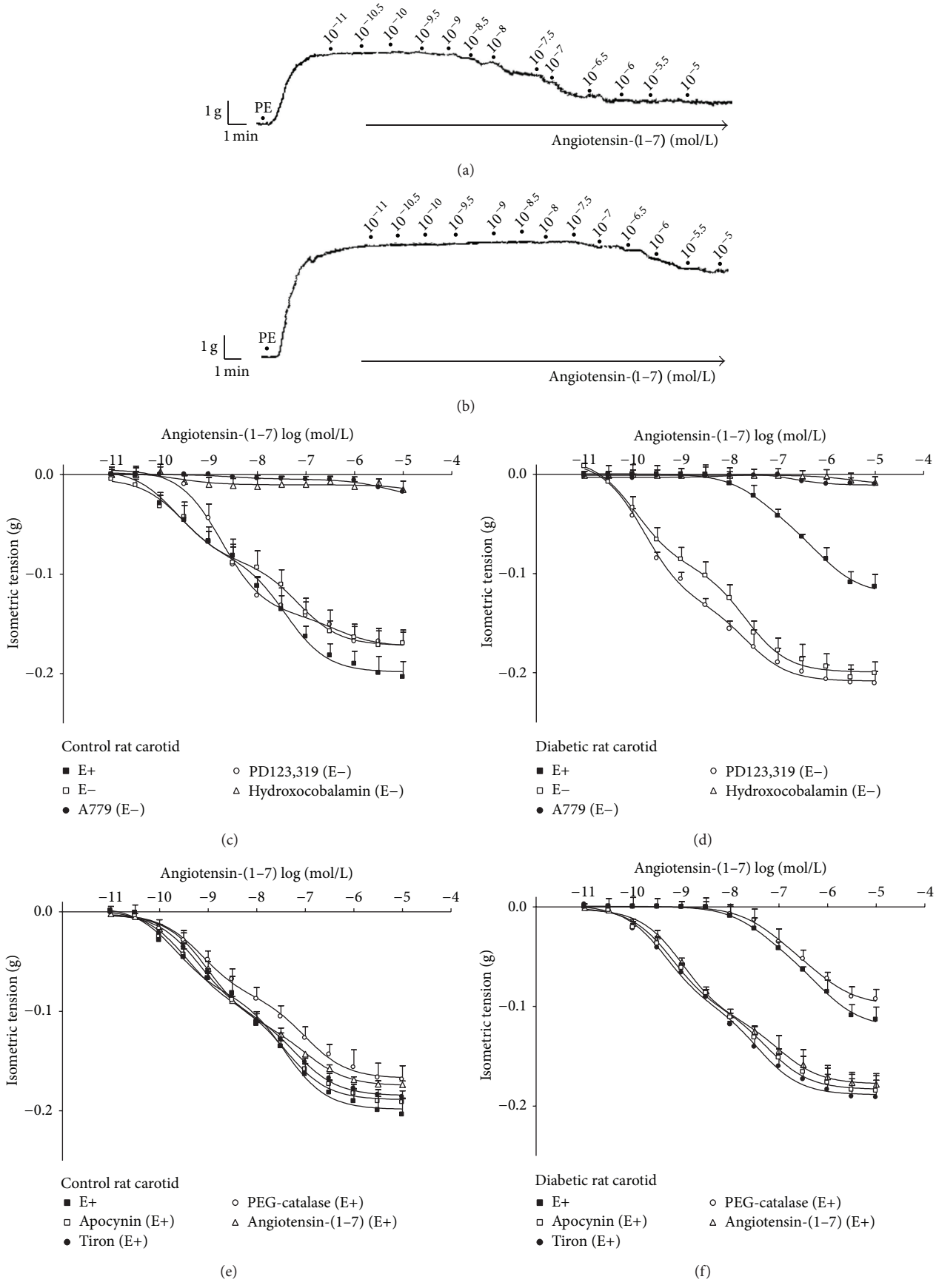
Figure 1: Concentration-response curves for angiotensin II in endothelium-intact (E+) or -denuded (E−) carotid rings from control or diabetic rats, over the precontraction induced by phenylephrine (PE). Representative traces from angiotensin II-evoked relaxation in E+ control (a) or diabetic (b) rat carotid. Effect of the *in vitro* pretreatment with DX600, A779, or PD123,319 in carotid rings from control (c) or diabetic (d) rats. Effect of the *in vitro* pretreatment with apocynin, tiron, PEG-catalase, or the chronic (*in vivo*) treatment with angiotensin-(1–7) in carotid rings from control (e) or diabetic (f) rats. Angiotensin II E_{max} in carotid arteries from control or diabetic rats before or after the *in vitro* pretreatment with DX600, A779, PD123,319 (g), apocynin, tiron, or PEG-catalase or the chronic treatment (*in vivo*) with angiotensin-(1–7) (h). The values are significantly different ($P < 0.01$; $n = 9$) from nonpretreated E+ (∗) or E− (∗∗) carotid rings from nontreated control rats, from nonpretreated E+ (#) or E− (##) carotid rings from nontreated diabetic rats, or from PEG-catalase pretreated E+ (†) carotid rings from nontreated control rats.

completely inhibited by A779, hydroxocobalamin (Figures 2(a), 2(c), and 2(g)) or L-NNA ($E_{max} = -0.03 \pm 0.005$ g, $n = 9$) ($P < 0.001$, *one-way ANOVA, Bonferroni post-hoc*), but not altered by L-NPA ($E_{max} = -0.18 \pm 0.011$ g, $n = 9$) or 1400 W ($E_{max} = -0.21 \pm 0.019$ g, $n = 9$) ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*). The magnitude of phenylephrine-induced precontraction in nontreated control rat carotid was not altered by endothelium removal (0.33 ± 0.031 g, $n = 9$) or the pretreatment with A779 (0.34 ± 0.028 g, $n = 9$), hydroxocobalamin (0.36 ± 0.034 g, $n = 9$), L-NNA (0.35 ± 0.031 g, $n = 9$), L-NPA (0.29 ± 0.027 g, $n = 9$), or 1400 W (0.27 ± 0.026 g, $n = 9$) ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*).

Diabetes impaired, but not abolished, the relaxant response induced by angiotensin-(1–7) in rat carotid ($E_{max} = -0.11 \pm 0.013$ g) over the precontraction induced by phenylephrine (0.51 ± 0.029 g, $n = 9$). This relaxant response was completely restored by endothelium removal (over again, this result required that the characterization of the mediators of angiotensin-(1–7)-induced relaxation was performed in endothelium-denuded carotid rings from diabetic rats, as well as from control rats, to compare the responses). In endothelium-denuded carotid rings from nontreated diabetic rats, the relaxant response evoked by angiotensin-(1–7) was completely inhibited by A779, hydroxocobalamin (Figures 2(b), 2(d), and 2(g)), or L-NNA ($E_{max} = -0.01 \pm 0.002$ g, $n = 9$) ($P < 0.001$, *one-way ANOVA, Bonferroni post-hoc*) but not altered by L-NPA ($E_{max} = -0.12 \pm 0.009$ g, $n = 9$) or 1400 W ($E_{max} = -0.10 \pm 0.007$ g, $n = 9$) ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*). The magnitude of phenylephrine-induced precontraction in nontreated diabetic rat carotid was not altered by endothelium removal (0.48 ± 0.035 g, $n = 9$) or the pretreatment with A779 (0.49 ± 0.025 g, $n = 9$), hydroxocobalamin (0.50 ± 0.041 g, $n = 9$), L-NNA (0.47 ± 0.023 g, $n = 9$), L-NPA (0.49 ± 0.032 g, $n = 9$), or 1400 W (0.51 ± 0.039 g, $n = 9$) ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*).

In endothelium-denuded carotid rings from nontreated control or diabetic rats, the maximum relaxation induced by angiotensin-(1–7) was lesser than that one induced by angiotensin II (Figures 2(a), 2(b), and 2(e)). However, the maximum relaxation induced by angiotensin II in losartan pretreated endothelium-intact carotid rings from nontreated control or diabetic rats had the same extent as the relaxation evoked by angiotensin-(1–7) in nonpretreated endothelium-intact carotid rings from nontreated control rat (Figure 3).

Although the relaxation induced by angiotensin-(1–7) has not been altered by losartan, apocynin, or tiron in endothelium-intact carotid rings from nontreated control rats, this response was completely restored by losartan, apocynin, or tiron in endothelium-intact carotid rings from nontreated diabetic rat. However, PEG-catalase, DCPIB, or the chronic treatment with angiotensin-(1–7) did not alter the relaxation induced by angiotensin-(1–7) in endothelium-intact carotid rings from nontreated control or diabetic rats. Similarly to the effects of losartan, apocynin, or tiron, the chronic treatment with angiotensin-(1–7) completely restored the relaxation induced by angiotensin-(1–7) in endothelium-intact carotid rings from diabetic rats (Figures 2(e), 2(f),



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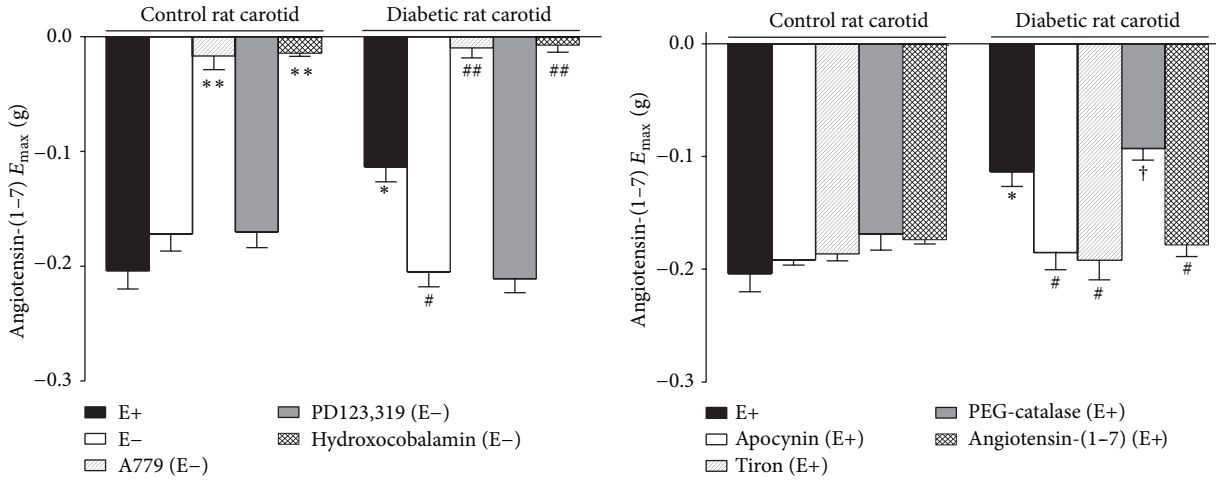

Figure 2: Concentration-response curves for angiotensin-(1–7) in endothelium-intact (E+) or -denuded (E−) carotid rings from control or diabetic rats, over the precontraction induced by phenylephrine (PE). Representative traces from angiotensin-(1–7)-evoked relaxation in E+ control (a) or diabetic (b) rat carotid. Effect of the *in vitro* pretreatment with A779, PD123,319 or hydroxocobalamin in carotid rings from control (c) or diabetic (d) rats. Effect of the *in vitro* pretreatment with apocynin, tiron, PEG-catalase, or the chronic (*in vivo*) treatment with angiotensin-(1–7) in carotid rings from control (e) or diabetic (f) rats. Angiotensin-(1–7) Emax in carotid from control or diabetic rats before or after the *in vitro* pretreatment with A779, PD123,319, hydroxocobalamin (g), apocynin, tiron, or PEG-catalase or the chronic (*in vivo*) treatment with angiotensin-(1–7) (h). The values are significantly different ($P < 0.01$; $n = 9$) from nonpretreated E+ (∗) or E− (∗∗) carotid rings from nontreated control rats, from nonpretreated E+ (#) or E− (##) carotid rings from nontreated diabetic rats, or from PEG-catalase pretreated E+ (†) carotid rings from nontreated control rats.

2(h), 3(b), and 3(d)). In control rat carotid, the magnitude of phenylephrine-induced precontraction was not altered by losartan (0.27 ± 0.022 g, $n = 9$), apocinin (0.26 ± 0.023 g, $n = 9$), tiron (0.29 ± 0.027 g, $n = 9$), PEG-catalase (0.31 ± 0.028 g, $n = 9$), angiotensin-(1–7) (0.30 ± 0.027 g, $n = 9$), or DCPIB (0.29 ± 0.021 g, $n = 9$). Also, in diabetic rat carotid, phenylephrine-induced precontraction was not altered by losartan (0.48 ± 0.033 g, $n = 9$), apocinin (0.51 ± 0.042 g, $n = 9$), tiron (0.50 ± 0.044 g, $n = 9$), PEG-catalase (0.49 ± 0.036 g, $n = 9$), angiotensin-(1–7) (0.47 ± 0.035 g, $n = 9$), or DCPIB (0.52 ± 0.039 g, $n = 9$), when compared to nonpretreated diabetic rat carotid ($P > 0.05$, *one-way ANOVA, Bonferroni post-hoc*).

3.3. Levels of O_2^-. The FI emitted by DHE-loaded endothelial cells from carotid arteries of nontreated diabetic rats was higher than the FI emitted by control samples. Tiron, apocynin, losartan, or the chronic treatment with angiotensin-(1–7) reduced the FI emitted by DHE-loaded samples from diabetic rats to the control levels obtained in the presence of these chemicals. DCPIB did not alter the FI emitted by DHE-loaded endothelial cells from carotid arteries of nontreated control (FI = 9,758.32 ± 561.29 U, $n = 5$) or diabetic (FI = 35,094.07 ± 915.43 U, $n = 5$) rats. The antioxidant effect produced by the chronic treatment with angiotensin-(1–7) in DHE-loaded samples from both control and diabetic rats was completely inhibited by the chronic treatment with A779 (Figure 4).

3.4. Levels of H_2O_2. The FI emitted by CDCF-DA-loaded endothelial cells from carotid arteries of nontreated diabetic rats was higher than the FI emitted by control samples. In CDCF-DA-loaded endothelial cells from carotid arteries of nontreated diabetic rats, PEG-catalase, tiron, apocynin, losartan, or the chronic treatment with angiotensin-(1–7) reduced the FI to the control levels obtained in the presence of PEG-catalase, apocynin, or losartan. DCPIB did not alter the FI emitted by CDCF-DA-loaded endothelial cells from carotid arteries of nontreated control (FI = 10,322.04 ± 517.81 U, $n = 5$) or diabetic (FI = 21,085.01 ± 639.43 U, $n = 5$) rats. The antioxidant effect produced by the chronic treatment with angiotensin-(1–7) in CDCF-DA-loaded samples from diabetic rats was completely inhibited by the chronic treatment with A779 (Figure 5).

3.5. ACE2, Angiotensin-(1–7), and Mas-Receptors Expression. In the muscular layer from nontreated diabetic rat carotid, ACE2 staining (39.05 ± 3.42%) was not altered, while angiotensin-(1–7) (2.09 ± 1.22%) or *Mas* receptors (17.63 ± 1.62%) staining was reduced when compared to nontreated control rat carotid (ACE2 = 47.28 ± 4.51%; angiotensin-(1–7) = 81.04 ± 6.29%; *Mas* = 92.37 ± 7.24%). *Mas* receptors staining was also detected in the endothelium from nontreated control rat carotid (64.57 ± 6.92%), but it was reduced in the endothelium from nontreated diabetic rat carotid (38.01 ± 2.79%) ($P < 0.001$, $n = 5$, *one-way ANOVA, Bonferroni post-hoc*) (Figure 6).

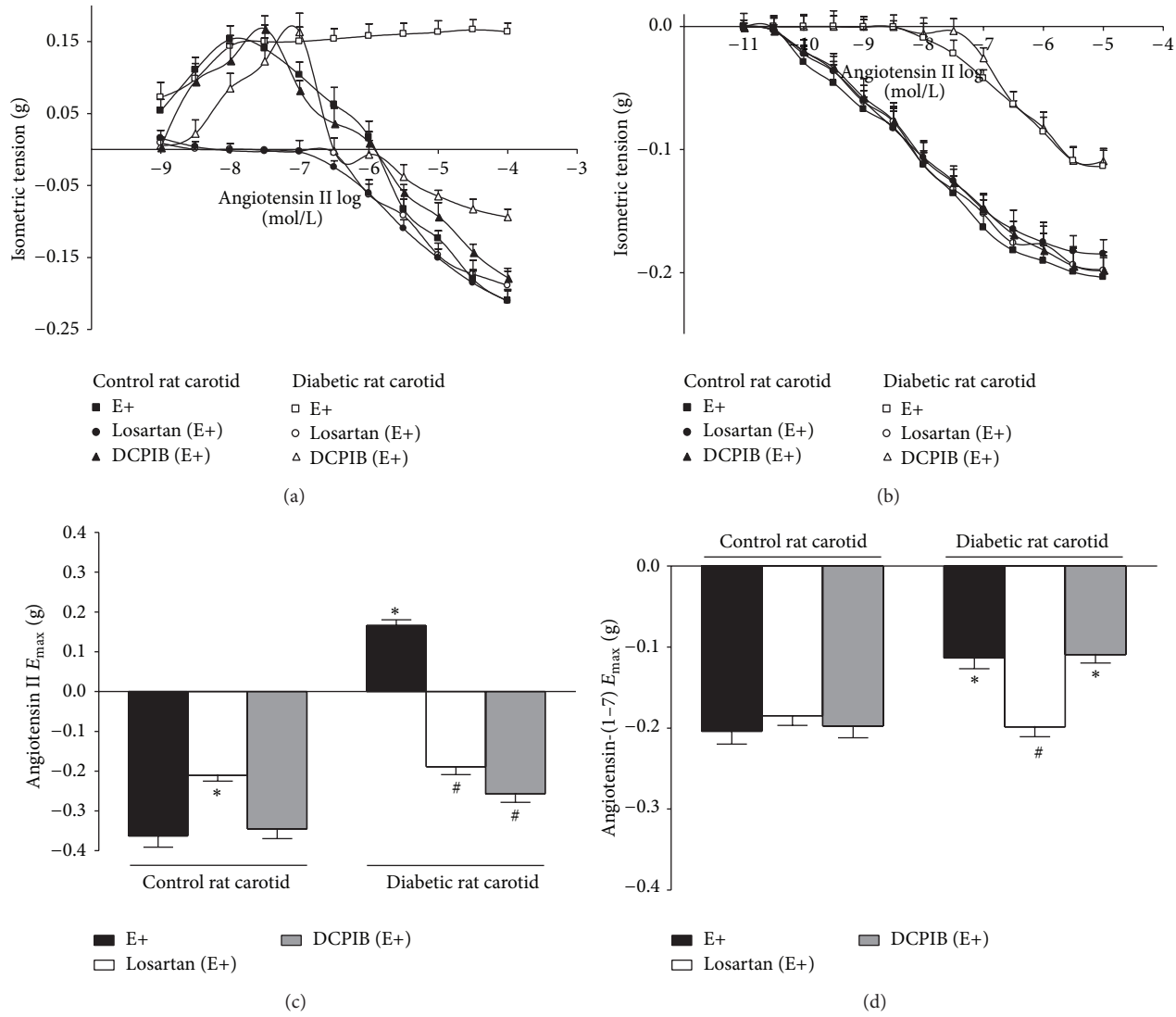

Figure 3: Concentration-response curves for angiotensin II or angiotensin-(1–7) in endothelium-intact (E+) carotid from control or diabetic rats after the *in vitro* pretreatment with losartan or DCPIB. Effect of the *in vitro* pretreatment with losartan or DCPIB on the relaxation induced by angiotensin II (a) or angiotensin-(1–7) (b). Angiotensin II E_{max} (c) or angiotensin-(1–7) E_{max} (d) before or after the *in vitro* pretreatment with losartan or DCPIB. The values are significantly different ($P < 0.01$; $n = 9$) from nonpretreated carotid rings (E+) from nontreated control (∗) or diabetic (#) rats.

The chronic treatment with angiotensin-(1–7) did not alter ACE2 expression in the muscular layer from control (42.37 ± 3.91%) or diabetic rat carotid (41.25 ± 4.14%). The chronic treatment with DX600 reduced angiotensin-(1–7) staining in the muscular layer from carotid arteries of control rats (4.01 ± 3.17%) to the levels obtained in carotid arteries from nontreated diabetic rats. On the other hand, the chronic treatment with DX600 did not alter angiotensin-(1–7) staining in the muscular layer from carotid arteries of diabetic rats (2.85±1.36%) when compared to nontreated diabetic rats ($P < 0.001$, $n = 5$, *one-way ANOVA, Bonferroni post-hoc*).

The chronic treatment with angiotensin-(1–7) did not alter the expression of *Mas* receptors neither in the endothelium from control (59.87 ± 5.76%) or diabetic (34.21 ± 3.07%) rat carotid nor in the muscular layer from control (87.23 ± 8.19%) or diabetic (14.95 ± 1.24%) rat carotid when compared to the carotid arteries from the respective nontreated groups ($P < 0.001$, $n = 5$, *one-way ANOVA, Bonferroni post-hoc*).

3.6. Blood Pressure, Carotid Blood Flow, and Carotid Resistance. Blood pressure was not different between nontreated or treated control or diabetic rats. In nontreated diabetic rats, carotid blood flow was reduced and carotid resistance was increased when compared to nontreated control rats. The chronic treatment of control rats with DX600 significantly reduced carotid blood flow and increased carotid resistance to the levels obtained in nontreated diabetic rats. The same effect was observed in carotid blood flow and carotid resistance from control rats that were treated with A779. In diabetic rats, carotid blood flow or carotid resistance

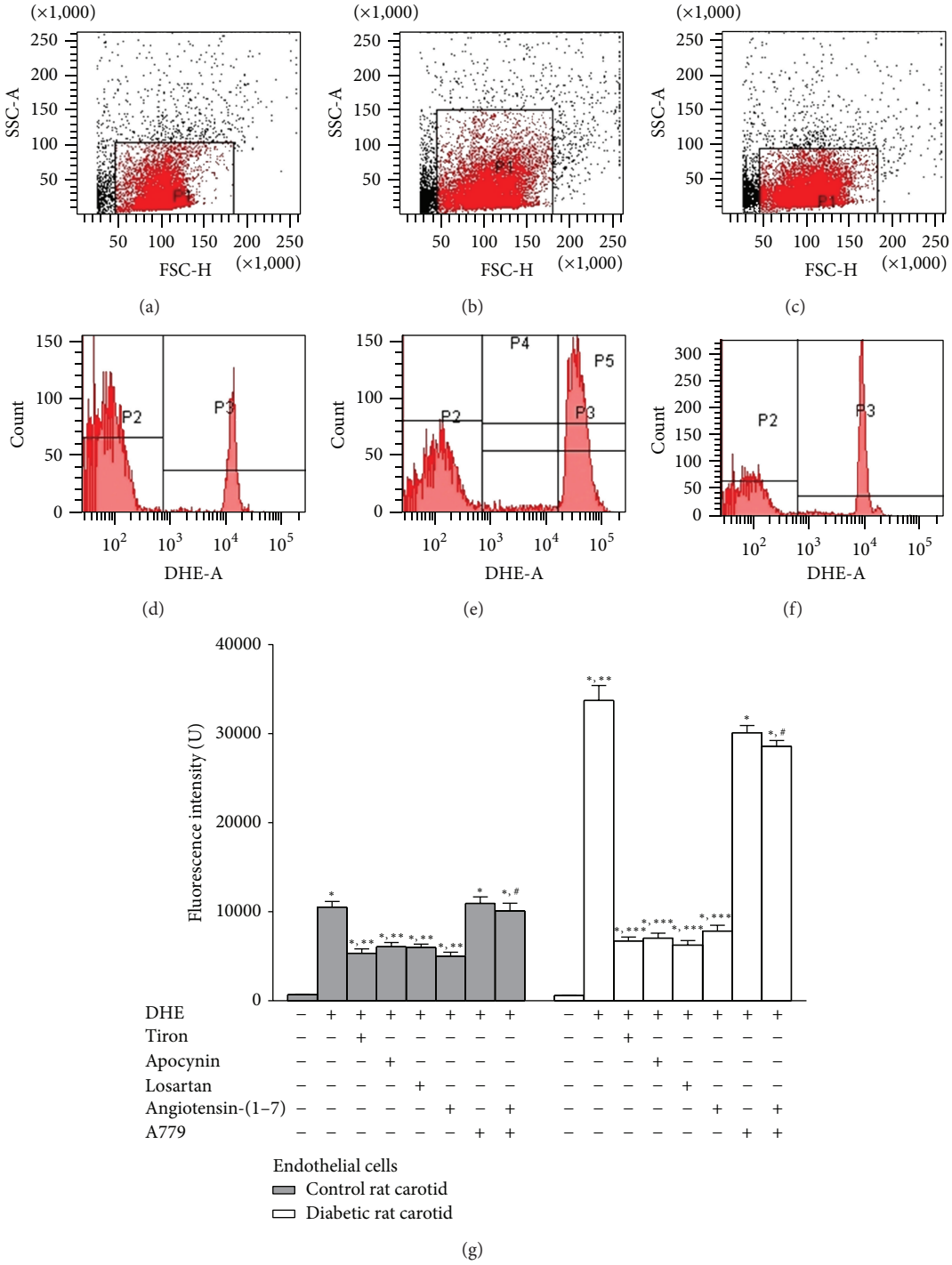

FIGURE 4: O_2^- levels in carotid endothelial cells from control or diabetic rats. Dot plots show the gates of carotid endothelial cells from nontreated control rats (a) or from nontreated (b) or angiotensin-(1–7)-treated (c) diabetic rats. Histograms show the fluorescence emitted by DHE-loaded carotid endothelial cells from nontreated control rats (d) or from nontreated (e) or angiotensin-(1–7)-treated diabetic rats (f). Effects of the *in vitro* pretreatment with tiron, apocynin, or losartan or the chronic (*in vivo*) treatment with angiotensin-(1–7) combined or not with A779 on the fluorescence emitted by DHE-loaded endothelial cells samples (g). The values are significantly different ($P < 0.01$; $n = 5$) from the respective blank samples from nontreated rats (∗), from nonpretreated DHE-loaded endothelial cells samples from nontreated control (∗∗) or diabetic (∗ ∗ ∗) rats, or from the respective DHE-loaded endothelial cells samples from angiotensin-(1–7)-treated rats (#).

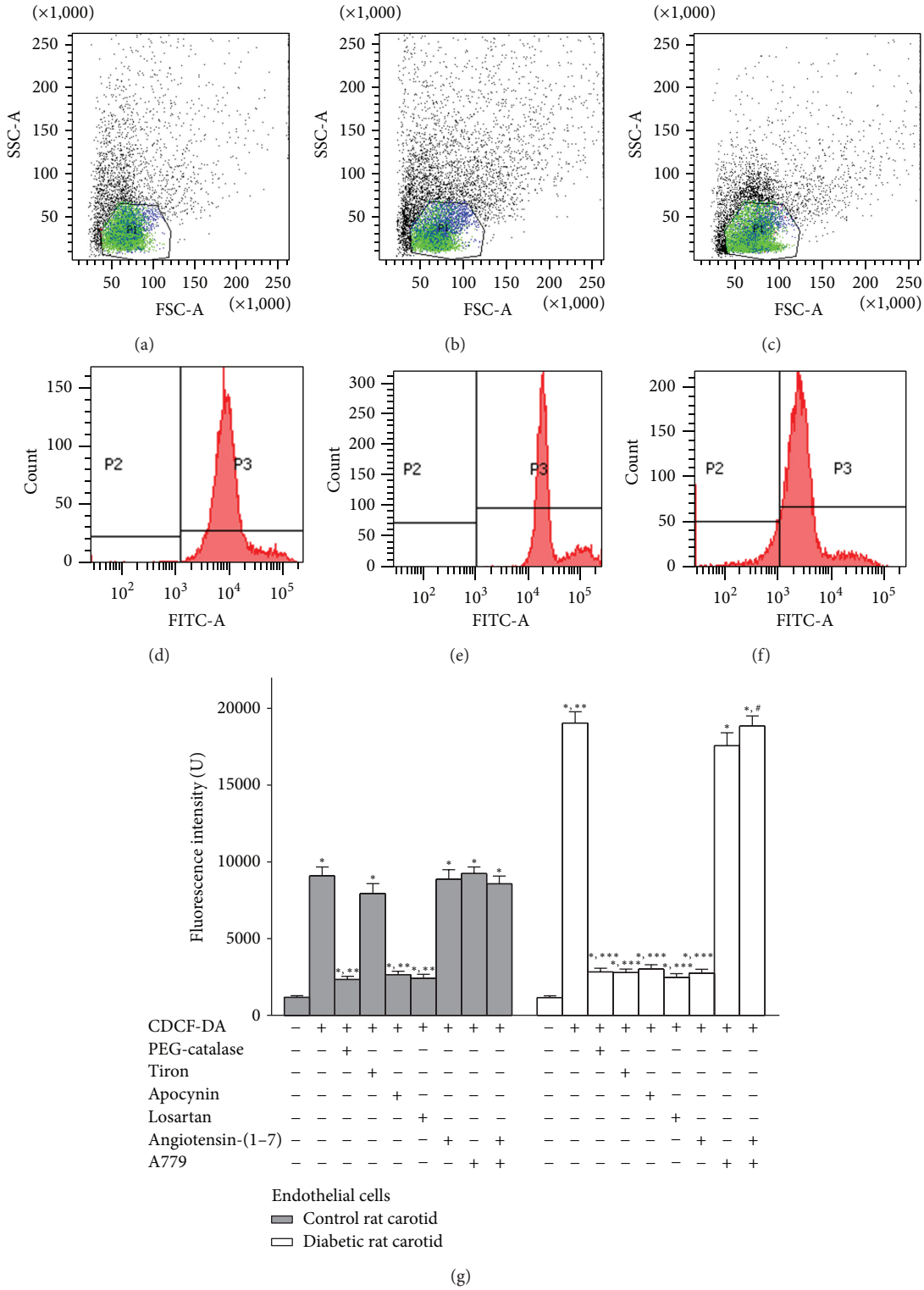

FIGURE 5: H_2O_2 levels in carotid endothelial cells from control or diabetic rats. Dot plots show the gates of carotid endothelial cells from nontreated control rats (a) or from nontreated (b) or angiotensin-(1–7)-treated diabetic rats (c). Histograms show the fluorescence emitted by CDCF-DA-loaded carotid endothelial cells from nontreated control rats (d) or from nontreated (e) or angiotensin-(1–7)-treated diabetic rats (f). Effects of the *in vitro* pretreatment with PEG-catalase, tiron, apocynin, or losartan or the chronic (*in vivo*) treatment with angiotensin-(1–7) combined or not with A779 on the fluorescence emitted by CDCF-DA-loaded endothelial cells samples (g). The values are significantly different ($P < 0.01$; $n = 5$) from the respective blank samples from nontreated rats (∗), from nonpretreated CDCF-DA-loaded endothelial cells samples from nontreated control (∗∗) or diabetic (∗ ∗ ∗) rats, or from CDCF-DA-loaded endothelial cells samples from angiotensin-(1–7)-treated diabetic rats (#).

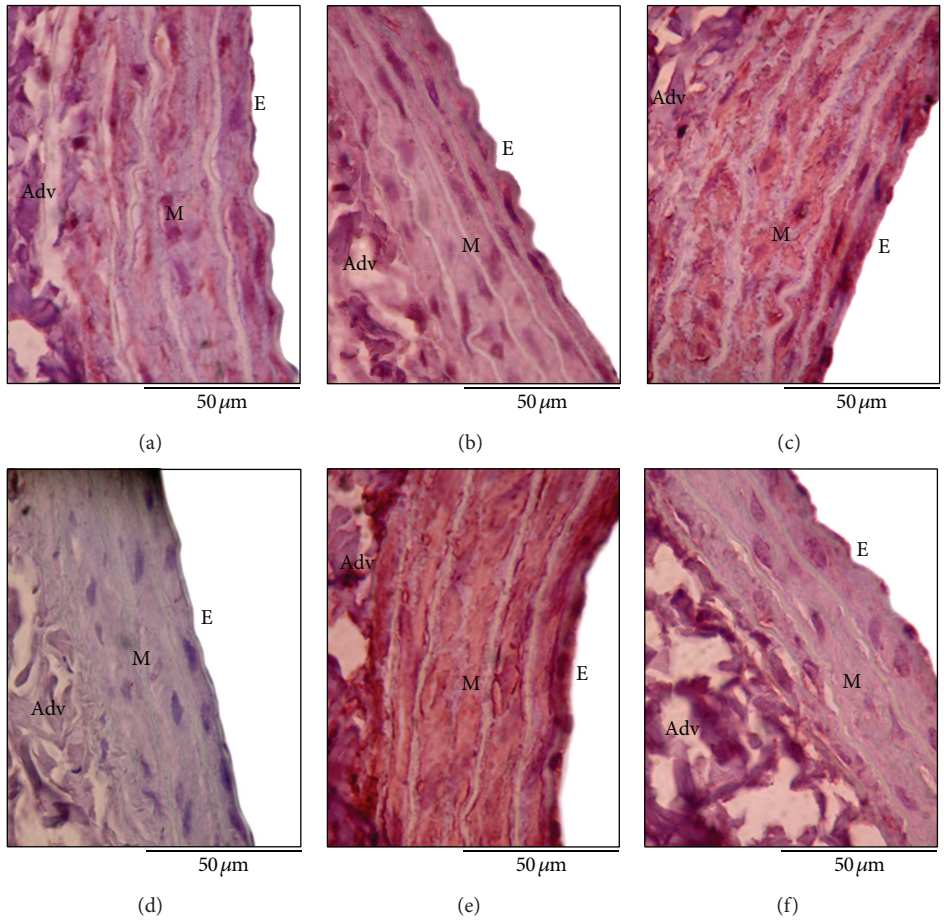

Figure 6: ACE2, angiotensin-(1–7) and *Mas* receptors staining in carotid arteries from nontreated control or diabetic rats. ACE2 expression in nontreated control (a) or diabetic (b) rat carotid, angiotensin-(1–7) levels in nontreated control (c) or diabetic (d) rat carotid, and *Mas* receptors expression in nontreated control (e) or diabetic (f) rat carotid. E: endothelium; M: media; Adv: adventitia. The immunostaining is denoted in red (magnification: 100x).

was not altered by the chronic treatment with DX600 or A779. The chronic treatment with angiotensin-(1–7) did not alter carotid blood flow or carotid resistance from control rats, but it restored carotid blood flow and carotid resistance from diabetic rats. A779 inhibited the effect of the chronic treatment with angiotensin-(1–7) on carotid blood flow and carotid resistance from diabetic rats (Table 3).

4. Discussion

The present study has three major new findings. The first one shows that type I-diabetes impairs the functionality of ACE2-angiotensin-(1–7)-*Mas* axis in rat carotid by a mechanism that involves the endothelial AT_1-activated NAD(P)H oxidase-driven generation of O_2^- and H_2O_2: while H_2O_2 derived from O_2^- dismutation inhibits ACE2 activity in generating angiotensin-(1–7) seemingly by activating $I_{Cl,SWELL}$, O_2^- inhibits the nitrergic vasorelaxant effect evoked by angiotensin-(1–7) upon *Mas* receptors activation. The second new finding shows that the impaired functionality of ACE2-angiotensin-(1–7)-*Mas* axis increases carotid resistance, which reduces carotid blood flow during type I-diabetes, highlighting the importance of the vasoprotective role assigned to the activation of this axis in both healthy and diseased conditions. Finally, the third new finding shows that the chronic treatment with angiotensin-(1–7) restores the functionality of carotid ACE2-angiotensin-(1–7)-*Mas* axis, what contributes to restore the carotid resistance and blood flow in diabetic conditions, by a mechanism that involves *Mas*-mediated antioxidant effects against the endothelial generation of O_2^- and H_2O_2 catalyzed by AT_1-activated NAD(P)H oxidase.

The functionality of vascular ACE2 (i.e., the activity of vascular ACE2 in generating angiotensin-(1–7)) was studied by relaxant curves induced by angiotensin II in rat carotid. This approach is supported by the evidences that angiotensin II-evoked relaxation in rat carotid is partially mediated by *Mas* receptors [19], seemingly upon angiotensin II hydrolysis into angiotensin-(1–7) and that the only enzyme able to convert angiotensin II into angiotensin-(1–7) in vascular tissues is ACE2 [17, 25]. Our findings show that angiotensin II-evoked relaxation curve in rat carotid is characterized by a

Table 3: Blood pressure (BP), carotid blood flow (F), and carotid resistance (R) from control or diabetic rats.

Rats	Cardiovascular parameters		
	BP (mmHg)	F (mL/min)	R (U)
Nontreated control	81.7 ± 5.4	6.11 ± 0.48	14.1 ± 2.3
Nontreated diabetic	99.2 ± 8.1	2.37 ± 0.21*	44.6 ± 3.2*
DX600-treated control	76.4 ± 6.2	2.09 ± 0.17*	41.9 ± 3.5*
DX600-treated diabetic	89.2 ± 7.5	2.13 ± 0.19	45.2 ± 4.2
Angiotensin-(1–7)-treated control	79.5 ± 6.1	5.76 ± 0.32	13.2 ± 2.9
Angiotensin-(1–7)-treated diabetic	97.0 ± 9.1	6.29 ± 0.51$^{\#}$	15.0 ± 2.1$^{\#}$
A779-treated control	81.1 ± 7.4	1.99 ± 0.14*	40.2 ± 4.3*
A779-treated diabetic	98.7 ± 8.3	2.44 ± 0.23	42.4 ± 4.1
Angiotensin-(1–7) + A779-treated control	75.6 ± 7.0	2.05 ± 0.19*,†	38.2 ± 3.8*,†
Angiotensin-(1–7) + A779-treated diabetic	99.5 ± 9.2	1.97 ± 0.11†	46.3 ± 4.7†

The values are significantly different ($P < 0.01$; $n = 5$) from nontreated control rats (∗), nontreated diabetic rats (#), or the respective rats treated with angiotensin-(1–7) (†).

biphasic response consisting in a residual AT_1-mediated contraction induced by nanomolar concentrations of angiotensin II (about 0.15 g), followed by an endothelium-independent relaxation induced by micromolar concentrations of this peptide (about −0.35 g). Our findings also show that DX600 completely abolishes the relaxation induced by angiotensin II without changing the residual contraction mediated by AT_1-receptors, which allows us to conclude that the relaxant response induced by angiotensin II in rat carotid occurs upon the ACE2-catalyzed hydrolysis of angiotensin II into angiotensin-(1–7). In turn, we found that losartan or A779 partially inhibits the relaxation triggered by angiotensin II: while losartan abrogates the complete relaxation of the residual contraction evoked by angiotensin II by inhibiting this contractile response, A779 does not alter neither the residual contraction evoked by angiotensin II nor the relaxation of this contractile response but abrogates the relaxation beyond the relaxant response that counteracts the residual contraction induced by angiotensin II. Thus, these findings allow us to divide the ACE2-dependent relaxation induced by angiotensin II in rat carotid into two components: (1) the losartan-sensitive relaxant response (about −0.15 g), which occurs upon the dissociation of angiotensin II from AT_1-receptors and the subsequent ACE2-catalyzed hydrolysis of angiotensin II into angiotensin-(1–7) and (2) the A779-sensitive relaxant response (about −0.2 g), which depends on the activation of *Mas* receptors by angiotensin-(1–7) generated from angiotensin II. Indeed, the vascular generation of angiotensin-(1–7) from angiotensin II is catalyzed by ACE2, which hydrolyzes only angiotensin II with high catalytic efficiency among the angiotensin peptides [17, 25]. Moreover, Tirapelli et al. [19] had already shown that *Mas* receptors partially mediate angiotensin II-induced relaxation in rat carotid.

Our results show that, similar to angiotensin II, angiotensin-(1–7) also evokes an endothelium-independent relaxation (about −0.2 g), which is completely mediated by *Mas*-receptors, NO, and endothelial NOS (*e*NOS) in rat carotid. In agreement with these findings, we also show that *Mas* receptors are mostly expressed in the muscular layer from rat carotid, supporting our functional evidence concerning the *Mas*-mediated endothelium-independent relaxation evoked by angiotensin-(1–7) in this vessel. Indeed, the vasorelaxation mediated by *Mas* receptors has mostly been described as a NOS-dependent nitrergic mechanism [19, 41–44], since the activation of these receptors by angiotensin-(1–7) leads to the phosphorylation of *e*NOS [45], which is also expressed in smooth muscle layer from rat carotid [9]. Another important aspect from our results is that the relaxant response induced by angiotensin-(1–7) had the same extent of the *Mas*-mediated (i.e., the A779-sensitive) relaxation evoked by angiotensin II in rat carotid, confirming that part of the relaxation induced by angiotensin II in this vessel is mediated by angiotensin-(1–7) upon the ACE2-catalyzed hydrolysis of the octapeptide.

Interestingly, our findings show that type I-diabetes inhibits the relaxant response induced by angiotensin II but only attenuates the *Mas*-mediated endothelium-independent relaxation evoked by angiotensin-(1–7) in rat carotid. Moreover, we observed that the residual relaxation evoked by angiotensin-(1–7) in diabetic rat carotid is mediated by NO and *e*NOS. Although the muscular expression of *e*NOS in this vessel is significantly reduced [9], recent evidences have shown that the acute stimulation with nano- to micromolar concentrations of angiotensin-(1–7) increases *e*NOS expression in rat cerebral ischemic tissues [46], spontaneously hypertensive rat ventricles [47], and human circulating fibrocytes [48]. These findings allow us to suggest that the generation of NO from muscular *e*NOS in diabetic rat carotid during angiotensin-(1–7)-evoked relaxation may be related to one of the following reasons: (1) the residual expression of *e*NOS at the muscular layer form diabetic rat carotid is enough to generate the NO levels that mediate this relaxant response or (2) the acute stimulation with nano- to micromolar concentrations of angiotensin-(1–7) may increase the expression of *e*NOS in this vessel, what would support the NO generation during the relaxation.

According to the present results, the inhibition of angiotensin II-induced relaxation in diabetic rat carotid, taken together with the findings concerning the unchanged

muscular expression of ACE2 and the reduced muscular levels of angiotensin-(1–7) in this vessel, allows us to strongly suggest that diabetes impairs the ACE2-catalyzed hydrolysis of angiotensin II into angiotensin-(1–7), but not the expression of the carboxypeptidase. This finding is confirmed when we show that the endogenous generation of angiotensin-(1–7) in rat carotid, which is catalyzed by ACE2, is lost in diabetic rats, since the chronic treatment with DX600 reduced angiotensin-(1–7) levels only in carotid arteries from normoglycaemic animals. Moreover, our functional results show that the diabetes-elicited inhibition of ACE2-catalyzed hydrolysis of angiotensin II in rat carotid (and thus, the inhibition of angiotensin II-induced relaxation) involves endothelial pathways completely mediated by AT_1-receptors, NAD(P)H oxidase, and O_2^- but partially mediated by H_2O_2. In turn, diabetes-elicited impairment of angiotensin-(1–7)-evoked relaxation involves endothelial mechanisms that are only mediated by AT_1-receptors, NAD(P)H oxidase, and O_2^-, but not by H_2O_2. In addition to these findings, we also observed that the *Mas*-mediated (A779-sensitive) relaxation induced by angiotensin II in PEG-catalase pretreated carotid rings from diabetic rat had the same extent as the relaxation induced by angiotensin-(1–7) in diabetic rat carotid (i.e., about −0.1 g). Taken together, these findings allow us to conclude that H_2O_2 generated from endothelial AT_1-activated NAD(P)H oxidase-derived O_2^- inhibits the ACE2-catalyzed hydrolysis of angiotensin II into angiotensin-(1–7), while AT_1-activated NAD(P)H oxidase-derived O_2^- impairs the nitrergic transduction pathways underlying *Mas* receptors activation in diabetic rat carotid. Confirming these data, we found an increase in the levels of H_2O_2 produced from AT_1-activated NAD(P)H oxidase-derived O_2^-, whose generation was also increased in endothelial cells from diabetic rat carotid. Indeed, type I-diabetes increases the endothelial generation of O_2^- derived from NAD(P)H oxidase in rat carotid [8, 9]. Moreover, the generation of H_2O_2 from O_2^- can occur by the dismutation of O_2^- [49]. In turn, upon AT_1-receptors activation by angiotensin II in endothelial cells, Nox1 subunit from vascular NAD(P)H oxidase can generate O_2^-, which is latter converted to H_2O_2 [50, 51]. Finally, type I-diabetes enhances the endothelial expression of AT_1-receptors and the reactive oxygen species generation mediated by these receptors in rat carotid, what explains the selective activation of endothelial NAD(P)H oxidase by AT_1-receptors in this vessel [8].

The cleavage of angiotensin II by somatic ACE2 can be reduced by increasing the physiological extracellular levels of Cl^- [30], due to the presence of a regulatory Cl^- binding site at the extracellular region of the carboxypeptidase [52]. Added to these findings, H_2O_2 generated from NAD(P)H oxidase-derived O_2^- can activate $I_{Cl,SWELL}$ in vascular smooth muscle cells, leading to the efflux of Cl^- and thus increasing Cl^- extracellular levels [31, 32]. Taken together, these findings prompted us to investigate the role of $I_{Cl,SWELL}$ in the modulation of ACE2-catalyzed hydrolysis of angiotensin II into angiotensin-(1–7) during the relaxation evoked by angiotensin II in diabetic rat carotid. Interestingly, we observed that the inhibition of $I_{Cl,SWELL}$ partially restored the relaxation evoked by angiotensin II, but not by angiotensin-(1–7), in diabetic rat carotid, similarly to the effect produced by the removal of H_2O_2. In addition, by showing that DCPIB did not alter O_2^- or H_2O_2 levels in endothelial cells from diabetic rat carotid, we excluded the hypothesis that the inhibition of $I_{Cl,SWELL}$ could lead to the scavenging of reactive oxygen species that modulate angiotensin II-evoked relaxation in this vessel. Thus, our data, supported by the findings from Rushworth et al. [30], Ren et al. [31], Matsuda et al. [32], and Towler et al. [52], strongly suggest that H_2O_2 generated from endothelial AT_1-activated NAD(P)H oxidase-derived O_2^- inhibits the ACE2-catalyzed hydrolysis of angiotensin II into angiotensin-(1–7) seemingly by activating $I_{Cl,SWELL}$ in smooth muscle cells from diabetic rat carotid. In turn, O_2^- derived from endothelial AT_1-activated NAD(P)H oxidase can impair nitrergic signaling pathways during type I-diabetic conditions, like the *Mas*-mediated relaxation evoked by angiotensin-(1–7) in rat carotid, by converting NO into peroxynitrite ($ONOO^-$) or by inducing to NOS uncoupling [53].

One of the most important aspects from our results is that the chronic treatment with angiotensin-(1–7) restores the functionality of ACE2-Angiotensin-(1–7)-*Mas* axis (i.e., both angiotensin II- and angiotensin-(1–7)-evoked relaxation) in diabetic rat carotid. According to our findings, this protective effect results from a *Mas*-mediated mechanism that inhibits the generation of O_2^- and H_2O_2 catalyzed by AT_1-activated NAD(P)H oxidase in endothelial cells from diabetic rat carotid. Moreover, we excluded the hypothesis that the restoration of ACE2-Angiotensin-(1–7)-*Mas* axis functionality in carotid arteries from angiotensin-(1–7)-treated diabetic rats could be resultant from a translational effect of enhancing ACE2 or *Mas* receptors expression, since they remained unaltered after the treatment. Thus, our results can be explained by the findings from Sampaio et al. [21], who described that the activation of *Mas* receptors by angiotensin-(1–7) inhibits the AT_1-mediated NAD(P)H oxidase assembly and the subsequent reactive oxygen species generation upon angiotensin II stimulation in endothelial cells.

Interestingly, even if type I-diabetes subregulates the endothelial expression of *Mas* receptors in rat carotid, the activation of this residual population of receptors during the chronic treatment with *Mas* agonists can trigger antioxidant effects that are enough to overcome the impairment of the local ACE2-angiotensin-(1–7)-*Mas* axis functionality. These findings have important implications to the therapeutic efficacy of *Mas* agonists on attenuating vascular dysfunctions and complications, since we showed that the activation of *Mas* receptors triggers a positive feedback over the ACE2-angiotensin-(1–7)-*Mas* axis when the functionality of this vasoprotective axis is impaired, even during diseases that subregulate the vascular expression of these receptors, such as type I-diabetes. Indeed, the chronic treatment of type I-diabetic rats with angiotensin-(1–7) or the nonpeptide *Mas* agonist AVE-0991 was efficacious in restoring the contractile and relaxation functions of mesenteric bed, carotid, and renal arteries [20].

Finally, we found that the chronic treatment with DX600 or A779 reduced carotid blood flow by increasing carotid

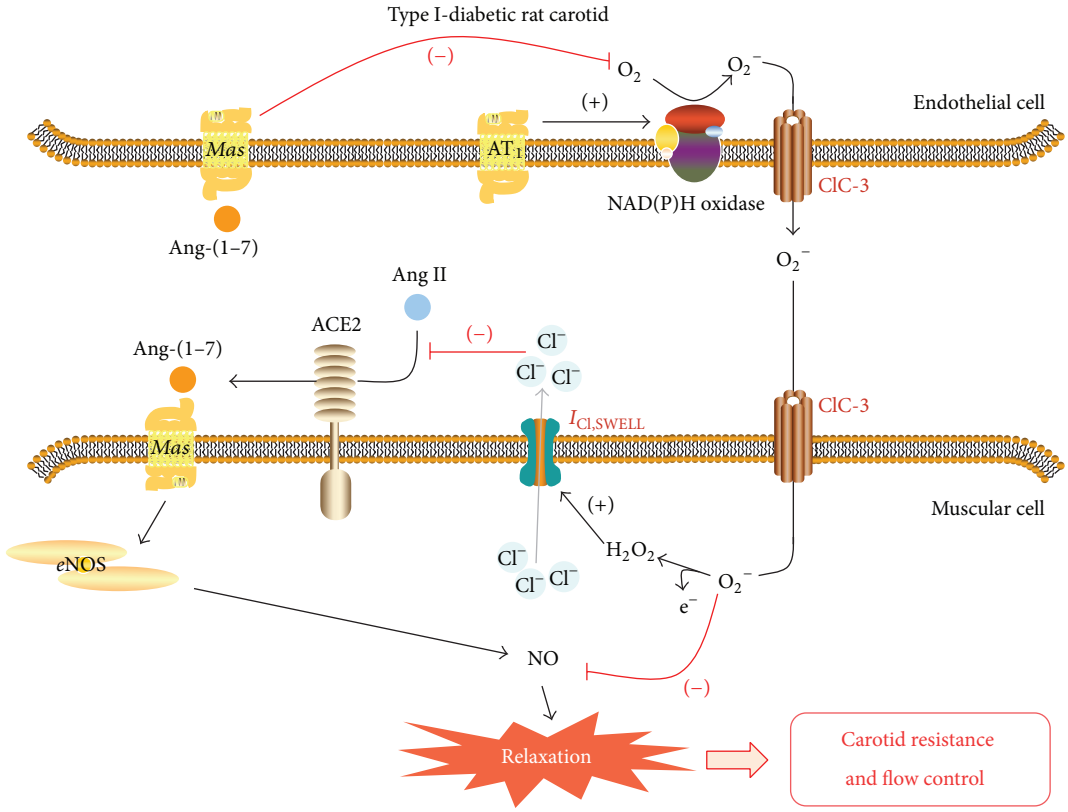

FIGURE 7: Conclusive graphical abstract. Endothelial AT_1-activated NAD(P)H oxidase-driven generation of O_2^- and H_2O_2 in carotid arteries from type I-diabetic rats impairs the functionality of the local vasoprotective ACE2-angiotensin-(1–7)-*Mas* axis, which in turn impairs carotid blood flow. In this mechanism, H_2O_2 derived from O_2^- dismutation inhibits ACE2 activity in generating angiotensin-(1–7) by activating $I_{Cl,SWELL}$, while O_2^- inhibits the nitrergic vasorelaxant effect evoked by angiotensin-(1–7) upon *Mas* receptors activation. The chronic treatment of diabetic rats with angiotensin-(1–7) restores the functionality of carotid ACE2-angiotensin-(1–7)-*Mas* axis by triggering a positive feedback on this axis, played by a residual population of endothelial *Mas*-receptors that blunts the endothelial AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species in rat carotid. *Mas*-mediated antioxidant effects evoked by the chronic treatment with angiotensin-(1–7) also restores carotid resistance and blood flow in diabetic rats, pointing the important contribution of the ACE2-angiotensin-(1–7)-*Mas* axis in maintaining carotid function.

resistance in normoglycaemic rats to the levels obtained with nontreated diabetic rats. On the other hand, carotid resistance and blood flow in diabetic rats were not altered after the chronic treatment with DX600 or A779. Moreover, while the chronic treatment with angiotensin-(1–7) did not alter carotid resistance and blood flow in normoglycaemic rats, it restored these parameters in diabetic rats, and this protective effect of angiotensin-(1–7) was inhibited when angiotensin-(1–7) was coadministrated with A779. Taken together, these findings point the vasoprotective role assigned to the ACE2-angiotensin-(1–7)-*Mas* axis functionality in maintaining carotid resistance and blood flow and confirm that the impairment of the functionality of this axis damages carotid function in diabetic conditions. Considering that carotid blood flow rate is determined, in a higher extent, by the resistance of smaller proximal arteries and arterioles (such as internal and external carotid arteries) than the carotid resistance, our findings allow us to suggest that the impairment of ACE2-angiotensin-(1–7)-*Mas* axis functionality reaches not only carotid artery but also the proximal resistance vessels.

5. Conclusions

This is the first study that shows that the endothelial AT_1-activated NAD(P)H oxidase-driven generation of O_2^- and H_2O_2 in carotid arteries from type I-diabetic rats impairs the functionality of the local vasoprotective ACE2-angiotensin-(1–7)-*Mas* axis, which in turn impairs carotid blood flow. In this mechanism, H_2O_2 derived from O_2^- dismutation inhibits ACE2 activity in generating angiotensin-(1–7) seemingly by activating $I_{Cl,SWELL}$, while O_2^- inhibits the nitrergic vasorelaxant effect evoked by angiotensin-(1–7) upon *Mas* receptors activation. Furthermore, we originally showed that the chronic treatment of diabetic rats with angiotensin-(1–7) restores the functionality of carotid ACE2-angiotensin-(1–7)-*Mas* axis by triggering a positive feedback on this axis, played by a residual population of endothelial *Mas*-receptors that blunts the endothelial AT_1-activated NAD(P)H oxidase-driven generation of reactive oxygen species in rat carotid. Finally, we found that the *Mas*-mediated antioxidant effects evoked by the chronic treatment with angiotensin-(1–7) also restores carotid resistance and blood flow in diabetic

rats, pointing the important contribution of the ACE2-angiotensin-(1–7)-*Mas* axis in maintaining carotid function (Figure 7). These findings have relevant implications to support the therapeutic efficacy of *Mas* agonists on preventing or attenuating diabetic endothelial dysfunction in carotid arteries and its underlying cerebrovascular complications by enhancing the vasoprotective role of the local ACE2-angiotensin-(1–7)-*Mas* axis.

Conflict of Interests

The authors declare there is no conflict of interests regarding the publication of this paper.

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References

[1] B. Glowinska-Olszewska, A. Hyniewicz, M. Jeznach et al., "Relationship between circulating endothelial progenitor cells and endothelial dysfunction in children with type 1 diabetes: a novel paradigm of early atherosclerosis in high-risk young patients," *European Journal of Endocrinology*, vol. 168, pp. 153–161, 2013.

[2] R. Dalla Pozza, A. Beyerlein, C. Thilmany et al., "The effect of cardiovascular risk factors on the longitudinal evolution of the carotid intima medial thickness in children with type 1 diabetes mellitus," *Cardiovascular Diabetology*, vol. 10, article 53, 2011.

[3] J. F. Polak, J.-Y. C. Backlund, P. A. Cleary et al., "Progression of carotid artery intima-media thickness during 12 years in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) study," *Diabetes*, vol. 60, no. 2, pp. 607–613, 2011.

[4] R. Hurks, M. J. Eisinger, I. Goovaerts et al., "Early endothelial dysfunction in young type 1 diabetics," *European Journal of Vascular and Endovascular Surgery*, vol. 37, no. 5, pp. 611–615, 2009.

[5] M. H. M. Yousif, I. F. Benter, and S. Akhtar, "The role of tyrosine kinase-mediated pathways in diabetes-induced alterations in responsiveness of rat carotid artery," *Autonomic and Autacoid Pharmacology*, vol. 25, no. 2, pp. 69–78, 2005.

[6] M. H. M. Yousif, I. F. Benter, N. Hares, H. Canatan, and S. Akhtar, "Phosphoinositide 3-kinase mediated signalling contributes to development of diabetes-induced abnormal vascular reactivity of rat carotid artery," *Cell Biochemistry and Function*, vol. 24, no. 1, pp. 13–22, 2006.

[7] Y. Shi and P. M. Vanhoutte, "Oxidative stress and COX cause hyper-responsiveness in vascular smooth muscle of the femoral artery from diabetic rats," *British Journal of Pharmacology*, vol. 154, no. 3, pp. 639–651, 2008.

[8] L. Pernomian, M. Santos Gomes, C. Baraldi Araujo Restini, L. Naira Zambelli Ramalho, C. Renato Tirapelli, and A. Maria De Oliveira, "The role of reactive oxygen species in the modulation of the contraction induced by angiotensin II in carotid artery from diabetic rat," *European Journal of Pharmacology*, vol. 678, no. 1–3, pp. 15–25, 2012.

[9] L. Pernomian, M. S. Gomes, F. M. A. Corrêa, C. B. A. Restini, L. N. Z. Ramalho, and A. M. de Oliveira, "Diabetes confers a vasoprotective role to the neurocompensatory response elicited by carotid balloon injury: consequences on contralateral carotid tone and blood flow," *European Journal of Pharmacology*, vol. 708, pp. 124–138, 2013.

[10] Z. J. Cheng, H. Vapaatalo, and E. Mervaala, "Angiotensin II and vascular inflammation," *Medical Science Monitor*, vol. 11, no. 6, pp. RA194–RA205, 2005.

[11] I. N. Migdalis, B. Gerolimou, G. Kozanidou, S. M. Hatzigakis, and K. D. Karmaniolas, "Effect of fosinopril sodium on early carotid atherosclerosis in diabetic patients with hypertension," *Journal of Medicine*, vol. 28, no. 5-6, pp. 371–380, 1997.

[12] M. Igarashi, A. Hirata, H. Yamaguchi et al., "Candesartan inhibits carotid intimal thickening and ameliorates insulin resistance in balloon-injured diabetic rats," *Hypertension*, vol. 38, no. 6, pp. 1255–1259, 2001.

[13] J.-P. Baguet, R. Asmar, P. Valensi, S. Nisse-Durgeat, and J. M. Mallion, "Effects of candesartan cilexetil on carotid remodeling in hypertensive diabetic patients: The MITEC Study," *Vascular Health and Risk Management*, vol. 5, pp. 175–183, 2009.

[14] M. Iwai and M. Horiuchi, "Devil and angel in the renin-angiotensin system: ACE-angiotensin II-AT1 receptor axis vs. ACE2-angiotensin-(1-7)-Mas receptor axis," *Hypertension Research*, vol. 32, no. 7, pp. 533–536, 2009.

[15] M. A. Fressato de Godoy, L. Pernomian, A. M. de Oliveira, and S. Rattan, "Biosynthetic pathways and the role of the MAS receptor in the effects of angiotensin-(1-7) in smooth muscle," *International Journal of Hypertension*, vol. 2012, Article ID 121740, 6 pages, 2012.

[16] S. R. Tipnis, N. M. Hooper, R. Hyde, E. Karran, G. Christie, and A. J. Turner, "A human homolog of angiotensin-converting enzyme: cloning and functional expression as a captopril-insensitive carboxypeptidase," *The Journal of Biological Chemistry*, vol. 275, no. 43, pp. 33238–33243, 2000.

[17] C. Vickers, P. Hales, V. Kaushik et al., "Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase," *The Journal of Biological Chemistry*, vol. 277, no. 17, pp. 14838–14843, 2002.

[18] R. A. S. Santos, A. C. Simoes e Silva, C. Maric et al., "Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8258–8263, 2003.

[19] C. R. Tirapelli, S. Y. Fukada, M. A. F. de Godoy, and A. M. de Oliveira, "Analysis of the mechanisms underlying the vasorelaxant action of angiotensin II in the isolated rat carotid," *Life Sciences*, vol. 78, no. 23, pp. 2676–2682, 2006.

[20] I. F. Benter, M. H. M. Yousif, C. Cojocel, M. Al-Maghrebi, and D. I. Diz, "Angiotensin-(1-7) prevents diabetes-induced cardiovascular dysfunction," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 1, pp. H666–H672, 2007.

[21] W. O. Sampaio, C. H. de Castro, R. A. S. Santos, E. L. Schiffrin, and R. M. Touyz, "Angiotensin-(1-7) counterregulates

angiotensin II signaling in human endothelial cells," *Hypertension*, vol. 50, no. 6, pp. 1093–1098, 2007.

[22] M. H. M. Yousif, G. S. Dhaunsi, B. M. Makki, B. A. Qabazard, S. Akhtar, and I. F. Benter, "Characterization of angiotensin-(1-7) effects on the cardiovascular system in an experimental model of type-1 diabetes," *Pharmacological Research*, vol. 66, pp. 269–275, 2012.

[23] L. Pernomian, M. S. Gomes, and A. M. de Oliveira, "Balloon catheter injury abolishes phenylephrine-induced relaxation in the rat contralateral carotid," *British Journal of Pharmacology*, vol. 163, no. 4, pp. 770–781, 2011.

[24] L. Pernomian, M. S. Gomes, C. B. A. Restini, A. S. Pupo, and A. M. de Oliveira, "Cross-talk with β2-adrenoceptors enhances ligand affinity properties from endothelial alpha1D-adrenoceptors that mediates carotid relaxation," *Journal of Pharmacology and Pharmacotherapeutics*, vol. 65, pp. 1337–1346, 2013.

[25] G. I. Rice, D. A. Thomas, P. J. Grant, A. J. Turner, and N. M. Hooper, "Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism," *Biochemical Journal*, vol. 383, no. 1, pp. 45–51, 2004.

[26] K. B. Pedersen, S. Sriramula, K. H. Chhabra, H. Xia, and E. Lazartigues, "Species-specific inhibitor sensitivity of angiotensin-converting enzyme 2 (ACE2) and its implication for ACE2 activity assays," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 301, no. 5, pp. R1293–R1299, 2011.

[27] M. J. Robertson, J. C. Barnes, G. M. Drew et al., "Pharmacological profile of GR117289 *in vitro*: a novel, potent and specific non-peptide angiotensin AT_1 receptor antagonist," *British Journal of Pharmacology*, vol. 107, no. 4, pp. 1173–1180, 1992.

[28] N. Decher, H. J. Lang, B. Nilius, A. Brüggemann, A. E. Busch, and K. Steinmeyer, "DCPIB is a novel selective blocker of I[CI,swell] and prevents swelling-induced shortening of guinea-pig atrial action potential duration," *British Journal of Pharmacology*, vol. 134, no. 7, pp. 1467–1479, 2001.

[29] W. Deng, L. Baki, and C. M. Baumgarten, "Endothelin signalling regulates volume-sensitive Cl- current via NADPH oxidase and mitochondrial reactive oxygen species," *Continuing Education in Anaesthesia, Critical Care and Pain*, vol. 88, no. 1, pp. 93–100, 2010.

[30] C. A. Rushworth, J. L. Guy, and A. J. Turner, "Residues affecting the chloride regulation and substrate selectivity of the angiotensin-converting enzymes (ACE and ACE2) identified by site-directed mutagenesis," *FEBS Journal*, vol. 275, no. 23, pp. 6033–6042, 2008.

[31] Z. Ren, F. J. Raucci Jr., D. M. Browe, and C. M. Baumgarten, "Regulation of swelling-activated Cl- current by angiotensin II signalling and NADPH oxidase in rabbit ventricle," *Cardiovascular Research*, vol. 77, no. 1, pp. 73–80, 2008.

[32] J. J. Matsuda, M. S. Filali, J. G. Moreland, F. J. Miller, and F. S. Lamb, "Activation of swelling-activated chloride current by tumor necrosis factor-α requires ClC-3-dependent endosomal reactive oxygen production," *The Journal of Biological Chemistry*, vol. 285, no. 30, pp. 22864–22873, 2010.

[33] D. Bonaventura, R. G. de Lima, J. A. Vercesi, R. S. da Silva, and L. M. Bendhack, "Comparison of the mechanisms underlying the relaxation induced by two nitric oxide donors: Sodium nitroprusside and a new ruthenium complex," *Vascular Pharmacology*, vol. 46, no. 3, pp. 215–222, 2007.

[34] D. Bonaventura, C. N. Lunardi, G. J. Rodrigues, M. A. Neto, and L. M. Bendhack, "A novel mechanism of vascular relaxation induced by sodium nitroprusside in the isolated rat aorta," *Nitric Oxide—Biology and Chemistry*, vol. 18, no. 4, pp. 287–295, 2008.

[35] J. Du, D. H. Daniels, C. Asbury et al., "Mitochondrial production of reactive oxygen species mediate dicumarol-induced cytotoxicity in cancer cells," *The Journal of Biological Chemistry*, vol. 281, no. 49, pp. 37416–37426, 2006.

[36] S. J. Robertson, K. D. Kania, S. B. Hladky, and M. A. Barrand, "P-glycoprotein expression in immortalised rat brain endothelial cells: comparisons following exogenously applied hydrogen peroxide and after hypoxia-reoxygenation," *Journal of Neurochemistry*, vol. 111, no. 1, pp. 132–141, 2009.

[37] H. Gao, U. Yallampalli, and C. Yallampalli, "Protein restriction to pregnant rats increases the plasma levels of angiotensin II and expression of angiotensin II receptors in uterine arteries," *Biology of Reproduction*, vol. 86, no. 3, article 68, 2012.

[38] A. C. Marshall, H. A. Shaltout, M. Nautiyal, J. C. Rose, M. C. Chappell, and D. I. Diz, "Fetal betamethasone exposure attenuates angiotensin-(1-7)-Mas receptor expression in the dorsal medulla of adult sheep," *Peptides*, vol. 44, pp. 25–31, 2013.

[39] J. F. Giani, J. G. Miquet, M. C. Muñoz et al., "Upregulation of the angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas receptor axis in the heart and the kidney of growth hormone receptor knock-out mice," *Growth Hormone & IGF Research*, vol. 22, pp. 224–233, 2012.

[40] D. Accorsi-Mendonça, F. M. A. Corrêa, T. B. Paiva, H. P. de Souza, F. R. M. Laurindo, and A. M. de Oliveira, "The balloon catheter induces an increase in contralateral carotid artery reactivity to angiotensin II and phenylephrine," *British Journal of Pharmacology*, vol. 142, no. 1, pp. 79–88, 2004.

[41] K. B. Brosnihan, P. Li, and C. M. Ferrario, "Angiotensin-(1-7) dilates canine coronary arteries through kinins and nitric oxide," *Hypertension*, vol. 27, no. 3, pp. 523–528, 1996.

[42] Y. Ren, J. L. Garvin, and O. A. Carretero, "Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles," *Hypertension*, vol. 39, no. 3, pp. 799–802, 2002.

[43] J.-M. Zhi, R.-F. Chen, J. Wang, X.-Y. Jiao, and R.-R. Zhao, "Comparative studies of vasodilating effects of angiotensin-(1-7) on the different vessels," *Acta Physiologica Sinica*, vol. 56, no. 6, pp. 730–734, 2004.

[44] V. S. Lemos, D. M. R. Silva, T. Walther, N. Alenina, M. Bader, and R. A. S. Santos, "The endothelium-dependent vasodilator effect of the nonpeptide Ang(1-7) mimic AVE 0991 is abolished in the aorta of *Mas*-knockout mice," *Journal of Cardiovascular Pharmacology*, vol. 46, no. 3, pp. 274–279, 2005.

[45] W. O. Sampaio, R. A. S. Dos Santos, R. Faria-Silva, L. T. Da Mata Machado, E. L. Schiffrin, and R. M. Touyz, "Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways," *Hypertension*, vol. 49, no. 1, pp. 185–192, 2007.

[46] Y. Zhang, J. Lu, J. Shi et al., "Central administration of angiotensin-(1-7) stimulates nitric oxide release and upregulates the endothelial nitric oxide synthase expression following focal cerebral ischemia/reperfusion in rats," *Neuropeptides*, vol. 42, no. 5-6, pp. 593–600, 2008.

[47] M. A. Costa, M. A. Lopez Verrilli, K. A. Gomez et al., "Angiotensin-(1-7) upregulates cardiac nitric oxide synthase in spontaneously hypertensive rats," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 299, no. 4, pp. H1205–H1211, 2010.

[48] K. Wang, X. Hu, C. Du, S. Tu, F. Zhang, and X. Xie, "Angiotensin-(1-7) suppresses the number and function of the circulating fibrocytes by upregulating endothelial nitric oxide synthase expression," *Molecular and Cellular Biochemistry*, vol. 365, no. 1-2, pp. 19–27, 2012.

[49] H. Mori and H. Iwahashi, "Superoxide dismutase enhanced the formation of hydroxyl radicals in a reaction mixture containing xanthone under UVA irradiation," *Bioscience, Biotechnology and Biochemistry*, vol. 71, no. 12, pp. 3014–3018, 2007.

[50] S. I. Dikalov, A. E. Dikalova, A. T. Bikineyeva, H. H. H. W. Schmidt, D. G. Harrison, and K. K. Griendling, "Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production," *Free Radical Biology and Medicine*, vol. 45, no. 9, pp. 1340–1351, 2008.

[51] T. Honjo, K. Otsui, R. Shiraki et al., "Essential role of NOXA1 in generation of reactive oxygen species induced by oxidized low-density lipoprotein in human vascular endothelial cells," *Endothelium*, vol. 15, no. 3, pp. 137–141, 2008.

[52] P. Towler, B. Staker, S. G. Prasad et al., "ACE2 X-ray structures reveal a large hinge-bending motion important for inhibitor binding and catalysis," *The Journal of Biological Chemistry*, vol. 279, no. 17, pp. 17996–18007, 2004.

[53] C. Szabo, "Role of nitrosative stress in the pathogenesis of diabetic vascular dysfunction," *British Journal of Pharmacology*, vol. 156, no. 5, pp. 713–727, 2009.

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Review Article

The Endothelium, A Protagonist in the Pathophysiology of Critical Illness: Focus on Cellular Markers

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The endotheliumis key in the pathophysiology of numerous diseases as a result of its precarious function in the regulation of tissue homeostasis. Therefore, its clinical evaluation providing diagnostic and prognostic markers, as well as its role as a therapeutic target, is the focus of intense research in patientswith severe illnesses. In the critically ill with sepsis and acute brain injury, the endothelium has a cardinal function in the development of organ failure and secondary ischemia, respectively. Cellular markers of endothelial function such as endothelial progenitor cells (EPC) and endothelialmicroparticles (EMP) are gaining interest as biomarkers due to their accessibility, although the lack of standardization of EPC and EMP detection remains a drawback for their routine clinical use. In this paper we will review data available on EPC, as a general marker of endothelial repair, and EMP as an equivalent of damage in critical illnesses, in particular sepsis and acute brain injury. Their determination has resulted in new insights into endothelial dysfunction in the critically ill. It remains speculative whether their determination might guide therapy in these devastating acute disorders in the near future.

1. Introduction

The endothelium forms the inner layer of blood and lymphatic vessels [1, 2]. Besides its mere role as a barrier between blood and tissue, the endothelial cell layer displays a myriad of physiological functions. Integrity of the endothelium is required for adequate deliverance of oxygen and nutrition to tissue and the migration of blood cells. Furthermore it plays a central role in coagulation and fibrinolysis, it regulates vascular tone and the formation of new blood vessels. As such the endothelium is a key regulator of homeostasis, for which continuous interaction with its environment is crucial. Its importance in the pathophysiology of not only cardiovascular, but also inflammatory and malignant diseases, is increasingly recognized.

The clinical evaluation of the endothelium has been thwarted by its location at the inner side of the vessels. The growing interest in the endothelium as a central player in numerous diseases has stimulated the development of a multitude of new circulating markers and in vivo evaluation techniques [1].

In this review we will focus on critically ill patients with sepsis and acute brain injury, both devastating conditions seen frequently in the intensive care unit. Sepsis and acute brain injury are characterized by secondary complications, that is, multiorgan failure and cerebral ischemia, respectively, which have enormous impact on outcome. Vascular dysregulation and endothelial dysfunction play a central role herein. As such, markers of endothelial dysfunction are of potential interest in determining prognosis.

2. Enumeration of Circulating Cellular Markers of Endothelial Dysfunction

During the last two decades cellular markers of endothelial repair and damage have emerged as potential noninvasive candidates for functional evaluation of the endothelium. In this overview, we highlight the role of endothelial progenitor cells (EPC) as a marker of endothelial repair and endothelial microparticles (EMP) as a measure for endothelial damage. We will briefly discuss their methods of detection. For thorough discussion on these matters we refer to recently published reviews [3–7].

Endothelial progenitor cells originate from the bone marrow and can differentiate into mature endothelial cells [3, 8]. In situations of ischemia and in case of inflammation, EPC repair damaged endothelium and help in creating capillary networks, in a direct and paracrine fashion [9]. Several humoral factors are implicated in their mobilization, differentiation, and homing such as vascular endothelial growth factor (VEGF), granulocyte macrophage colony forming factor, stromal derived factor 1α (SDF 1α), erythropoietin (EPO), amongst others [10]. Despite a multitude of papers published on EPC in various diseases, their definition remains a point of debate. The confusion on EPC definitions originates from the various techniques used for their detection having poor inter-method agreement (different types of cell culture techniques and flow cytometry) [3, 4, 11]. In cell culture techniques we discriminate two types, that is, short-term culture identifying early outgrowth EPC and long-term cultures isolating truly proliferating cells with endothelial fate [3, 4]. Since the first rather identifies hematopoietic cells involved in angiogenesis and the last are very elaborative, long-term cultures up to 30 days necessitating large amounts of blood and resulting in low colony counts, flow cytometry is at this moment the preferred technique for their detection in clinical studies. However there is a lack of any specific phenotypic marker for EPC to use in flow cytometry. Asahara et al. were the first to describe putative EPC, and they used a combination of CD34, a hematopoietic and progenitor cell marker, and flk-1/KDR, a receptor for VEGF important for homing of EPC and expressed on endothelial cells [8]. Both markers, however, are rather aspecific and as such are also expressed on mature circulating endothelial cells. For this reason Peichev et al. added CD133, a stem cell marker to better differentiate true EPC [12]. A drawback of using these triple positive cells, as circulating marker of endothelial function, is that their number is so low that enumeration becomes less reliable [3]. Furthermore it has been shown that these cells do develop into hematopoietic and not endothelial colonies [13]. EPC defined as CD34 and KDR positive cells have been most widely evaluated in clinical studies and have proven to be implicated in angiogenesis and endothelial repair in vivo [4, 9, 14]. Hence our research group prefers to use these cells as markers of endothelial repair, keeping in mind that this is a heterogeneous group of cells possessing an overlapping phenotype with endothelial cells and hematopoietic progenitors [6].

Endothelial microparticles (EMP) originate through vesiculation of the endothelial cell membrane upon cell activation, damage, or apoptosis [38]. EMP are membrane particles smaller than 1 μm, which contain oxidized phospholipids and proteins characteristic of endothelial cells. Surface antigens vary with the microparticle generating process; CD31+, CD105+, and Annexin V+ EMP are generated mainly during apoptosis, while CD62E, CD54, and CD106 expression are mostly seen when E are released upon activation [39, 40]. For their detection flow cytometry is the mainly used and mostly studied technique [41]. It has been shown that preanalytical and analytical heterogeneity amongst various research groups has led to differing results [38, 41, 42]. At this moment efforts are made for analytical and preanalytical standardization for flow cytometric detection of microparticles [43, 44]. Another difficulty for the use of EMP as biomarker in the critically ill is the possible interference with lipid-rich solutions [45]. The use of propofol and total parenteral nutrition in these patients could lead to secondary lipid accumulation negatively influencing the number of microparticles detected by flow cytometry. EMP are increasingly used as a marker of endothelial damage, especially in cardiovascular disorders, but growing evidence also indicates that EMP have an important modulating role in inflammation, coagulation, and vascular function [5, 38].

Multiparameter analysis for the evaluation of endothelial function is emerging as a valuable ex vivo tool for assessment of endothelial function [46], in addition to its potential to further unravel the pathophysiology of endothelial disruption in several disease conditions [7].

3. The Endothelium in Sepsis: The Orchestrator of Organ Failure

In one of four patients hospitalized at the intensive care unit severe sepsis is the reason for admission [47, 48]. Sepsis is defined as the systemic inflammatory response syndrome to an infection [49]. It is a devastating disorder that can progress to severe sepsis with the development of organ dysfunction, septic shock when hypotension is unresponsive to fluid resuscitation, and eventually multiorgan failure and death [49]. These stages of severity form a continuum in which patients evolve during their disease and treatment. The chance of survival decreases with the progression of the sepsis syndrome over this continuum. Hospital mortality in sepsis varies between 14 and 45% in Europe [47, 48]. Despite important advances in microbiological and supportive therapy, mortality has only slightly improved during the last decades [47]. Organ failure is the major cause of death in sepsis patients [50]. This is further supported by the finding that the number of organs that failed correlates with short-term mortality [51] and that the therapeutic improvement of organ failure early in sepsis improves survival [52].

3.1. Endothelial Function in Sepsis. The pathophysiology of sepsis is complex. Being multifactorial and heterogeneous among patients is two of the main characteristics of sepsis [53]. Sepsis is caused by a systemic maladaptive response of the host to an invading microorganism. Under normal conditions, infection triggers a local inflammatory reaction associated with an antiinflammatory response, local activation of the coagulation process together with a systemic acute

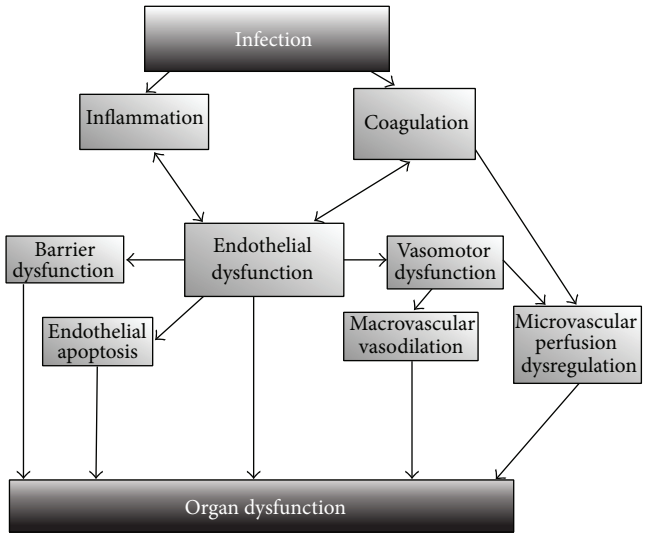

Figure 1: Endothelial dysfunction in sepsis. In sepsis the reaction that has the aim of containing the infection derails and leads to a proinflammatory, procoagulant situation and endothelial dysfunction, finally resulting in the development of organ failure.

phase and neurohumoral response. All these reactions are finely tuned with the purpose of containing the infection with minimal damage. The complex interaction of these responses leading to the conquest of the infection in one case could derail and lead to sepsis in others. The exact factors leading to sepsis are not completely understood but are host and microorganism dependent. The endothelium has a central position in orchestrating both the physiologic and pathological host response to infection due to its regulation of cellular permeability, coagulation, and vascular blood flow [54].

In the development of distant organ dysfunction, macro- and microvascular dysfunction play an important role. Macrovascular dysfunction during sepsis constitutes of 2 major effects: hyperdynamic shock due to hypovolemia caused by venous and arterial vasodilation at the macrovascular level and capillary leak and cardiac depression [55]. On the other hand it has been shown that tissue hypoperfusion remains despite normalization of macrocirculatory derangements, underlining the importance of additional microvascular derangements and mitochondrial dysfunction in sepsis [56, 57]. At microvascular level there is heterogeneity in flow, stopped flow, and decreased density of perfused capillaries [56]. As such, in sepsis the microcirculation is unable to adequately regulate microvascular perfusion to local oxygen demand.

The endothelium is a key component in the development of these macro- and microcirculatory disturbances in sepsis (see Figure 1). Activation of the endothelium leads to a procoagulant and proinflammatory condition, a disrupted barrier and an abnormal vascular tone [2]. In sepsis there is a direct destruction of the endothelial barrier [2, 58], and an increased amount of circulating endothelial cells has been shown in patients with septic shock [59]. The vasomotor regulation is also hampered in sepsis. More in particular there is an imbalance between vasodilator and vasoconstrictor signaling molecules leading to an impaired vasomotor tone. Despite increased concentration of circulating catecholamines in sepsis there is a decreased vascular response to these factors [60]. On the other hand a disturbed endothelial mediated vasodilation has also been shown at macrovascular and microvascular level [61–64].

Evaluating circulating endothelial markers in patients with sepsis has evolved from circulating endothelial adhesion-molecules to cellular markers of endothelial repair and damage.

3.2. Endothelial Progenitor Cells in Sepsis. Several groups have investigated EPC in sepsis but their role has not yet been unequivocally defined [15–22] (see Table 1). Observational studies found an increased percentage of circulating EPC enumerated by flow cytometry in highly selected patients with sepsis [15, 19, 20], while experimental studies, that is, the administration of LPS in healthy volunteers and a MODS model in pigs showed a decreased number [17, 18]. At our own center, we found a decreased absolute number of EPC in a heterogeneous group of severe sepsis patients compared to healthy volunteers [22]. Furthermore, while Becchi et al. found an increased number of EPC in severe sepsis versus sepsis patients, Rafat et al. found a positive correlation between EPC number and survival [15, 20]. Our data are in line with the last findings, with lower numbers of absolute EPC in patients with increasing sequential organ failure assessment (SOFA) score, a measure of severity of organ failure, during the first week after sepsis. Differences in study population, expressing results as percentage of peripheral blood mononuclear cells (PBMC) (which are decreased in sepsis) versus absolute numbers; methodology (isolated PBMC versus whole blood) and the different phenotypes studied can explain these opposing findings. Despite these contradictory findings, the functional impairment of EPC seems indisputable in sepsis [16–19, 22]. All studies, case-control and experimental, describe decreased proliferative or migratory capacities of EPC [16, 17, 19, 21, 22]. Data on the exact role of EPC in vascular repair during sepsis are scarce. Lam et al., for example, showed that EPC transplantation in a rabbit ARDS model decreased endothelial dysfunction, maintained the alveolocapillary membrane, and reduced inflammation [65]. As mentioned before, numerous humoral factors influence EPC mobilization, differentiation, and homing; therefore, EPC are an important therapeutic target to stimulate endothelial repair in sepsis. Several therapeutic strategies that focus on sepsis-related endothelial dysfunction have been shown to influence EPC [66], for example, statins, shown to increase EPC number and ameliorate their functional capacity, that is, decreasing senescence and improving proliferation [67, 68].

3.3. Endothelial Microparticles in Sepsis. Several studies, case-control human studies as well as animal models, have explored EMP in sepsis, with differing results (see Table 2) [22, 23, 25–27, 29]. EMP were found to be increased in patients with sepsis by some research groups [25, 26, 29], while others found a decreased or equal number [22, 23].

Table 1: Overview endothelial progenitor cells in sepsis.

Study group	Study type	Phenotype EPC	Main findings
Becchi et al. [15]	Case-control sepsis ($n = 24$)	CD34+ KDR+ in isolated PBMC CD34+ KDR+ CD133+ in isolated CD34+ cells	(i) Increased % EPC the first 72 h after sepsis (ii) EPC severe sepsis ≫ sepsis
Cribbs et al. [16]	Case-control sepsis ($n = 86$)	CFU-EPC	(i) Decreased CFU-EPC in sepsis (ii) Inversely associated with SOFA
Luo et al. [17]	MODS model in pigs ($n = 20$)	CD133+ CD34+ in WB CFU-EPC Migration to VEGF	Decreased EPC, CFU-EPC and migratory function in MODS
Mayr et al. [18]	LPS in healthy volunteers ($n = 32$)	CD34+ KDR+ CD133+ EPC in WB CFU-EPC	(i) Decrease in EPC number with nadir at 6 h post LPS (ii) Decreased CFU-EPC nadir 4 h after LPS
Patschan et al. [19]	Case-control sepsis ($n = 40$)	KDR+ CD133+ in isolated PBMC CFU-EPC	(i) Increased % EPC in sepsis (ii) Decreased CFU-EPC in sepsis
Rafat et al. [20]	Case-control sepsis ($n = 32$)	CD34+ KDR+ CD133+ in isolated PBMC	(i) Increased % EPC in sepsis (ii) Lower % EPC in nonsurvivors
Schlichting et al. [21]	Case-control severe sepsis ($n = 18$)	CFU-EPC	No difference
van Ierssel et al. [22]	Case-control severe sepsis ($n = 30$)	CD34+ KDR+ in WB Migration to SDF-1α and VEGF	(i) Decreased absolute number (ii) Decreased migratory capacity (iii) Impending organ dysfunction the first week was associated with lower EPC and a trend to impaired migration

CFU-EPC: EPC colony forming units; EPC: endothelial progenitor cells, LPS: lipopolysaccharides; MODS: multiorgan dysfunction syndrome; PBMC: peripheral blood mononuclear cells; SDF-1α: stromal derived factor 1 α; SOFA: sequential organ failure assessment; VEGF: vascular endothelial growth factor; WB: whole blood.

These inconsistent results may result from a lack of pre- and analytical standardization of microparticle (MP) detection, the different phenotypes studied, and differences in study population. In contrast to the interpretation in cardiovascular diseases, where an elevation of EMP is considered a marker of endothelial dysfunction, the number of EMP is positively related to survival and inversely correlated with the SOFA-score in patients with sepsis [29]. Since it is becoming more and more clear that microparticles are more than simple markers of endothelial damage or activation, their interpretation as marker of endothelial dysfunction is less unambiguous. As such it has been shown that the general pool of MP in septic patients is protective against vascular hyporeactivity in vitro, increasing the response to 5-HT in vitro while not affecting endothelium-dependent vasodilation [25]. Mortaza et al., on the other hand, found that injection of MP from septic rats induced vasodilatory shock in healthy animals [24]. MP also have been implicated in hypercoagulability and inflammation [23, 26, 29]. Finally Pérez-Casal et al. found increased numbers of MP bearing endothelial protein C receptor (EPCR) of endothelial and monocytic origin in patients treated with recombinant protein C [28]. These MP decreased apoptosis and reduced permeability in endothelial cells in an APC dependent way, a confirmation of earlier in vitro findings [69]. At this moment the knowledge on EMP functions in sepsis is too scarce to clarify their role in the development of organ failure.

4. The Endothelium as Key Player in Secondary Cerebral Ischemia after Acute Brain Injury

Acute brain injury, more in particular subarachnoid hemorrhage (SAH) and traumatic brain injury (TBI), is devastating neurological events which have an important socioeconomical impact. The development of secondary cerebral ischemia is an important prognostic factor in both SAH and TBI [70–72]. It develops in 8–12% and 20–30% of patients after TBI and SAH, respectively, mostly within the first 2 weeks after the insult [70, 71, 73, 74].

4.1. Endothelial Function and Secondary Cerebral Ischemia after Subarachnoid Bleeding. In SAH the concepts of delayed cerebral ischemia (DCI) and cerebral vasospasm have been well studied and clearly defined (see Figure 2) [75]. While previously macrovascular cerebral vasospasm was thought key for the development of DCI, it is now accepted to be a multifactorial process of which the exact underlying mechanisms are not yet completely unraveled [75]. As such it has been repetitively shown that macrovascular vasospasms are not a condition sine quo non to develop DCI, and on the other hand not all vasospasms will lead to the development of DCI [74]. Other mechanisms such as microvascular dysfunction, disturbed autoregulation, thromboembolism, and cortical spreading depression have been implicated in

Table 2: Overview endothelial microparticles in sepsis.

Study group	Study design	Detection method	Phenotype EMP	Main findings
Joop et al. [23]	Case-control MODS and sepsis ($n = 9$)	Flow cytometry isolated MP frozen samples	CD62E+/ Annexin V+ CD144+/ Annexin V+	(i) Lower number CD62E+ EMP (ii) Unchanged number CD144+ EMP
Mortaza et al. [24]	Rat cecal ligation and puncture model	Flow cytometry PFP	CD54+/ Annexin V+	(i) Unchanged EMP in sepsis (ii) Septic MP caused vasoplegic shock in healthy rats
Mostefai et al. [25]	Case-control sepsis ($n = 36$) mouse model: injection of septic MP	Flow cytometry PFP frozen samples	CD146+	(i) Increased EMP in sepsis (ii) Septic MP induced increased responsiveness to vasoconstrictors in aortic rings
Nieuwland et al. [26]	Case-control meningococcal sepsis ($n = 7$)	Flow cytometry isolated MP	CD62E+/ Annexin V+	Nonsignificant increase in sepsis
Ogura et al. [27]	Case-control severe SIRS ($n = 28$, sepsis = 12)	Flow cytometry PRP	CD54+ CD31+	EMP increased in sepsis
Pérez-Casal et al. [28]	Case control study of APC treated sepsis patients	Flow cytometry isolated MP	CD13+ EPCR+	Increased CD13+ EPCR+ MP
Soriano et al. [29]	Case control severe sepsis ($n = 35$)	Flow cytometry PPP	CD31+ CD42b−	(i) EMP higher in severe sepsis (ii) EMP higher in survivors (iii) Negative correlation with SOFA on D2 and D3
van Ierssel et al. [22]	Case-control severe sepsis ($n = 26$)	Flow cytometry PPP	CD31+ CD42b−	Unchanged number of EMP versus healthy controls

EMP: endothelial microparticles; MODS: multiorgan dysfunction syndrome; MP: microparticle; PFP: platelet free plasma; PPP: platelet poor plasma; PRP: platelet rich plasma; SOFA: sequential organ dysfunction assessment.

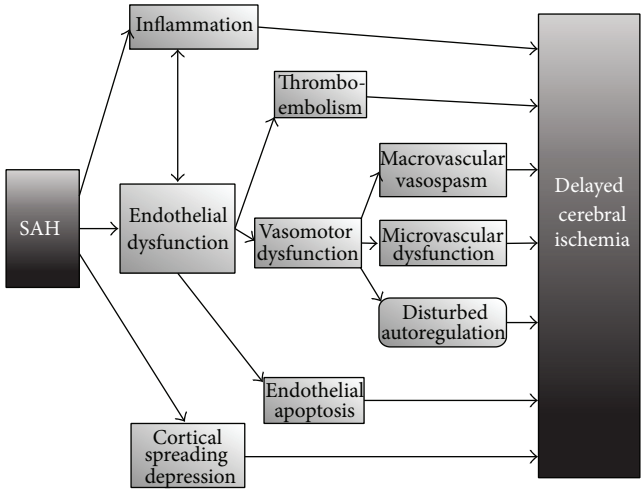

Figure 2: Endothelial dysfunction in SAH. SAH: subarachnoid hemorrhage. In subarachnoid hemorrhage, the development of delayed cerebral ischemia is a multifactorial process in which besides macrovascular vasospasm; thromboembolism, disturbed autoregulation, microvascular dysfunction, and cortical spreading depression are involved. Endothelial dysfunction is a key factor in the development of these processes. It is not clarified yet if local and general inflammation are causal factors or bystanders in the development of secondary ischemia.

its development [76–78]. The endothelial function, in all its aspects, is a crucial factor in these proposed mechanisms. It plays a central role in the formation of microthrombi by regulating vasoconstriction and expressing of P-selectin [79]. Furthermore it has been shown that cerebral vascular reactivity and cerebral autoregulation are disturbed after SAH [77, 80]. The endothelium plays an important role in modulating vascular tone. As such both endothelial derived vasodilators (e.g., NO) and vasoconstrictors (e.g., endothelin) are important in the development of macrovascular cerebral vasospasm and in microvascular dysfunction [81, 82]. Moreover, cerebral endothelial cell apoptosis has been documented after experimental SAH [83]. The role of inflammation in the development of ischemia is not clarified yet, but the endothelium is important in the regulation of diapedesis of leucocytes and local inflammation [81, 84].

4.2. Endothelial Function and Secondary Ischemia after Traumatic Brain Injury. In traumatic brain injury (TBI), on the other hand, the concept of posttraumatic cerebral ischemia is less well studied and understood. This can be explained by the fact that patients with TBI are a very heterogeneous group and that besides the primary cerebral injury other extra-cerebral processes may cause secondary damage [70, 71, 85]. The mechanisms involved are mechanical compression,

Table 3: Overview endothelial progenitor cells in acute brain injury, SAH, and TBI.

Study group	Study type	Phenotype EPC	Main findings
Liang et al. [30]	Case-control unruptured intracranial aneurysm ($n = 24$)	CFU-EPC Migration to VEGF	Decreased proliferative and migratory capacity of EPC
Liu et al. [31]	Case-control TBI ($n = 29$)	CD34+ CD133+ in isolated PBMC	Decreased EPC in TBI, steady increase from day 5–7 with peak day 7
Liu et al. [32]	Case-control TBI ($n = 84$)	CD34+ CD133+ in isolated PBMC	(i) Decreased EPC 24–48 h after TBI, increase to day 7 (ii) Non-survivors lower EPC
Wei et al. [33]	Case-control ruptured cerebral aneurysm ($n = 14$)	CD34+ CD133+ Isolated PBMC	(i) Decreased number of EPC in patients (ii) Increase after coiling with a peak at day 14
Wei et al. [34]	Case-control cerebral aneurysm ($n = 56$, ruptured $n = 35$)	CD34+ CD133+ CD34+ KDR+ in isolated PBMC Migration to VEGF	(i) Both EPC phenotypes reduced in cerebral aneurysm (ii) Impaired migration and increased of EPC in cerebral aneurysm

EPC: endothelial progenitor cells; PBMC: peripheral blood mononuclear cells; VEGF: vascular endothelial growth factor; TBI: traumatic brain injury.

hypotension, direct vascular injury, thromboembolism, and posttraumatic cerebral vasospasm; moreover, distinguishing these is difficult (see Figure 3). The appearance of posttraumatic cerebral vasospasms has been related to the presence of traumatic subarachnoid blood but has also been reported in the absence of a traumatic SAH [86]. These findings suggest that besides the mechanisms important in the development of vasospasm after spontaneous SAH, other processes are involved after TBI, such as direct stretching and mechanical irritation [86]. Furthermore the relation between cerebral vasospasm and the development of secondary ischemia is still a point of debate, and there are only few prospective studies on this matter [86, 87]. Besides macrovascular changes, the microvasculature is also involved. As such in animal experiments diffuse loss of microvasculature networks and capillary density after TBI were found [88]. Increased VEGF expression, indicating a possible role for neovascularization [88], and impaired cerebral endothelium-dependent cerebral vascular responses have also been documented [89].

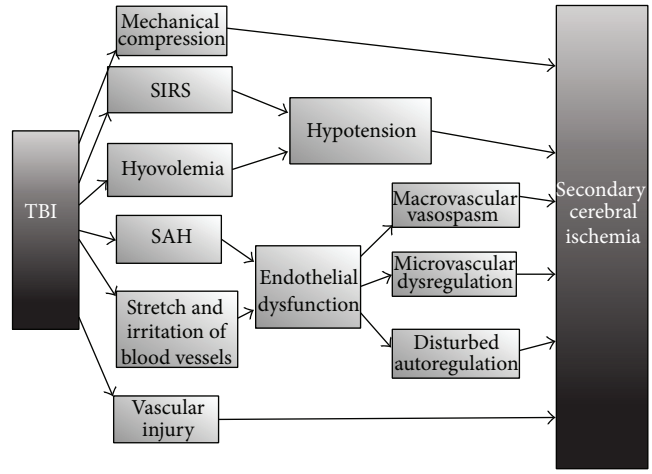

Figure 3: Development of delayed cerebral ischemia after traumatic brain injury. SAH: subarachnoid hemorrhage; SIRS: systemic inflammatory response syndrome; TBI: traumatic brain injury. In traumatic brain injury the exact pathophysiology of secondary ischemia is not completely clarified. Besides cerebral mechanisms, extracerebral processes are also involved such as hypotension. On the other hand the endothelium seems to be a central player in its development.

4.3. Endothelial Progenitor Cells after Acute Brain Injury. Until now research on markers of endothelial function in SAH and TBI has mostly focused on circulating endothelial adhesion molecules and markers of endothelial activation [90–92], both of which are increased in patients developing secondary cerebral ischemia. Endothelial progenitor cells show a biphasic response after traumatic brain injury; after an initial decrease they peak 7 days after the insult (see Table 3) [31]. Furthermore they have been associated with an improved outcome after TBI [32]. In patients with cerebral aneurysm a decreased number of EPC also has been shown, possibly related to patients' risk factors (e.g., smoking and hypertension) [34]. Our group also enumerated EPC in patients with SAH and TBI and confirmed the finding of a decreased number of EPC initially after the insult. (van Ierssel S.H., unpublished results) publication) Furthermore an impaired functional capacity of EPC was seen [30, 34]. After endovascular coiling of ruptured aneurysm there is a rapid increase of EPC with a peak at 14 days after rupture [33]. At this moment, we are not aware of studies on the relation between EPC and DCI or posttraumatic cerebral ischemia. The exact role of EPC in vascular repair after acute brain injury has not been studied yet. In a rat model of traumatic brain injury Wang et al. looked at the role of atorvastatin [93]. They found an increased number of EPC and enhanced cerebral angiogenesis, together with an improved functional outcome in treated rats. These results again show the importance of EPC as a possible therapeutic target.

4.4. Endothelial Microparticles in Acute Brain Injury. Few researchers have looked at the evolution of endothelial

Table 4: Overview on endothelial microparticles in acute brain injury, SAH, and TBI.

Study group	Study population	Detection method	Phenotype EMP	Main findings
Lackner et al. [35]	Case-control spontaneous SAH ($n = 20$)	Flow cytometry plasma	CD105+/Annexin V+ or − CD62E+/Annexin V+ or − CD54+/Annexin V+ or − CD106+/Annexin V+ or −	(i) Increased number of all EMP phenotypes studied in SAH versus healthy (ii) In patients with Doppler detected cerebral vasospasm increased CD105+/Annexin V+ and CD62E+/Annexin V+ (iii) CD105+/Annexin V+ associated with cerebral infarction
Morel et al. [36]	Case-control TBI ($n = 16$)	Capture technique PFP and CSF	Annexin V+ CD31+	(i) Increased MP number in plasma and CSF at D0, decreased D3, D5, D10 (ii) High proportion of EMP
Sanborn et al. [37]	Case-control SAH ($n = 22$)	Flow cytometry Frozen plasma samples	CD146+/Annexin V+	(i) Elevated EMP after SAH, and remained high until D10 (ii) Negative correlation EMP and infarction at D14

CSF: cerebral spinal fluid; EMP: endothelial microparticles; MP: microparticle, PFP: Platelet free plasma; SAH: subarachnoid hemorrhage; TBI: traumatic brain injury.

microparticles after SAH and TBI (see Table 4) [35–37]. They all found an increased number of EMP after acute brain injury, in line with the common use of EMP as markers of endothelial damage [38]. With regard to the development of secondary ischemia, the relation seems more ambiguous. While Lackner et al. found an increased number of CD105+ Annexin+ EMP early after the insult, Sanborn et al. found a decreased number of CD146+ EMP at Day 1 in patients developing DCI [35, 37]. The different populations that were studied can explain these opposing results, as well as the variable pre- and analytical methods used and the variances in phenotype of EMP studied. At this moment there are no data available on the exact functional role of EMP after acute brain injury.

5. Conclusion

The endothelium seems to be a central actor in the development of organ failure in sepsis and secondary ischemia after acute brain injury, as illustrated here for SAH and TBI. The exact role of markers of repair (EPC) and injury (EMP), however, needs further clarification. Nevertheless the importance of both organ failure and secondary ischemia in the prognosis of these devastating disorders explains the craving for adequate prognostic and therapeutic clues and hence the interest in the endothelium makes common sense.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] J. E. Deanfield, J. P. Halcox, and T. J. Rabelink, "Endothelial function and dysfunction: testing and clinical relevance," *Circulation*, vol. 115, no. 10, pp. 1285–1295, 2007.

[2] W. C. Aird, "The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome," *Blood*, vol. 101, no. 10, pp. 3765–3777, 2003.

[3] G. P. Fadini, D. Losordo, and S. Dimmeler, "Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use," *Circulation Research*, vol. 110, no. 4, pp. 624–637, 2012.

[4] M. C. Yoder, "Human endothelial progenitor cells," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 7, Article ID a006692, 2012.

[5] F. Dignat-George and C. M. Boulanger, "The many faces of endothelial microparticles," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 1, pp. 27–33, 2011.

[6] E. M. van Craenenbroeck, A. H. van Craenenbroeck, S. van Ierssel et al., "Quantification of circulating $CD34^+/KDR^+/CD45^{dim}$ endothelial progenitor cells: analytical considerations," *International Journal of Cardiology*, vol. 167, no. 5, pp. 1688–1695, 2013.

[7] D. Burger and R. M. Touyz, "Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells," *Journal of the American Society of Hypertension*, vol. 6, no. 2, pp. 85–99, 2012.

[8] T. Asahara, T. Murohara, A. Sullivan et al., "Isolation of putative progenitor endothelial cells for angiogenesis," *Science*, vol. 275, no. 5302, pp. 964–967, 1997.

[9] P. Madeddu, C. Emanueli, E. Pelosi et al., "Transplantation of low dose $CD34^+KDR^+$ cells promotes vascular and muscular regeneration in ischemic limbs," *The FASEB Journal*, vol. 18, no. 14, pp. 1737–1739, 2004.

[10] B. R. Everaert, E. M. van Craenenbroeck, V. Y. Hoymans et al., "Current perspective of pathophysiological and interventional effects on endothelial progenitor cell biology: focus on Pi3K/AKT/eNOS pathway," *International Journal of Cardiology*, vol. 144, no. 3, pp. 350–366, 2010.

[11] E. M. F. van Craenenbroeck, V. M. A. Conraads, D. R. van Bockstaele et al., "Quantification of circulating endothelial progenitor cells: a methodological comparison of six flow cytometric approaches," *Journal of Immunological Methods*, vol. 332, no. 1-2, pp. 31–40, 2008.

[12] M. Peichev, A. J. Naiyer, D. Pereira et al., "Expression of VEGFR-2 and AC133 by circulating human $CD34^+$ cells identifies a population of functional endothelial precursors," *Blood*, vol. 95, no. 3, pp. 952–958, 2000.

[13] J. Case, L. E. Mead, W. K. Bessler et al., "Human $CD34^+AC133^+$ $VEGFR\text{-}2^+$ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors," *Experimental Hematology*, vol. 35, no. 7, pp. 1109–1118, 2007.

[14] G. P. Fadini, I. Baesso, M. Albiero, S. Sartore, C. Agostini, and A. Avogaro, "Technical notes on endothelial progenitor cells: ways to escape from the knowledge plateau," *Atherosclerosis*, vol. 197, no. 2, pp. 496–503, 2008.

[15] C. Becchi, S. Pillozzi, L. P. Fabbri et al., "The increase of endothelial progenitor cells in the peripheral blood: a new parameter for detecting onset and severity of sepsis," *International Journal of Immunopathology and Pharmacology*, vol. 21, no. 3, pp. 697–705, 2008.

[16] S. K. Cribbs, D. J. Sutcliffe, W. R. Taylor et al., "Circulating endothelial progenitor cells inversely associate with organ dysfunction in sepsis," *Intensive Care Medicine*, vol. 38, no. 3, pp. 429–436, 2012.

[17] T. H. Luo, Y. Wang, Z. M. Lu et al., "The change and effect of endothelial progenitor cells in pig with multiple organ dysfunction syndromes," *Critical Care*, vol. 13, no. 4, article no. R118, 2009.

[18] F. B. Mayr, A. O. Spiel, J. M. Leitner, C. Firbas, W. Sieghart, and B. Jilma, "Effects of low dose endotoxemia on endothelial progenitor cells in humans," *Atherosclerosis*, vol. 195, no. 1, pp. e202–e206, 2007.

[19] S. A. Patschan, D. Patschan, J. Temme et al., "Endothelial progenitor cells (EPC) in sepsis with acute renal dysfunction (ARD)," *Critical Care*, vol. 15, no. 2, article R94, 2011.

[20] N. Rafat, C. Hanusch, P. T. Brinkkoetter et al., "Increased circulating endothelial progenitor cells in septic patients: correlation with survival," *Critical Care Medicine*, vol. 35, no. 7, pp. 1677–1684, 2007.

[21] D. E. Schlichting, A. B. Waxman, L. A. O'Brien et al., "Circulating endothelial and endothelial progenitor cells in patients with severe sepsis," *Microvascular Research*, vol. 81, no. 2, pp. 216–221, 2011.

[22] S. H. van Ierssel, E. M. van Craenenbroeck, V. Y. Hoymans, C. J. Vrints, V. M. Conraads, and P. G. Jorens, "Endothelium dependent vasomotion and in vitro markers of endothelial repair in patients with severe sepsis: an observational study," *PLoS ONE*, vol. 8, no. 8, Article ID e69499, 2013.

[23] K. Joop, R. J. Berckmans, R. Nieuwland et al., "Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms," *Thrombosis and Haemostasis*, vol. 85, no. 5, pp. 810–820, 2001.

[24] S. Mortaza, M. C. Martinez, C. Baron-Menguy et al., "Detrimental hemodynamic and inflammatory effects of microparticles originating from septic rats," *Critical Care Medicine*, vol. 37, no. 6, pp. 2045–2050, 2009.

[25] H. A. Mostefai, F. Meziani, M. L. Mastronardi et al., "Circulating microparticles from patients with septic shock exert protective role in vascular function," *American Journal of Respiratory and Critical Care Medicine*, vol. 178, no. 11, pp. 1148–1155, 2008.

[26] R. Nieuwland, R. J. Berckmans, S. McGregor et al., "Cellular origin and procoagulant properties of microparticles in meningococcal sepsis," *Blood*, vol. 95, no. 3, pp. 930–935, 2000.

[27] H. Ogura, H. Tanaka, T. Koh et al., "Enhanced production of endothelial microparticles with increased binding to leukocytes in patients with severe systemic inflammatory response syndrome," *Journal of Trauma—Injury, Infection and Critical Care*, vol. 56, no. 4, pp. 823–830, 2004.

[28] M. Pérez-Casal, V. Thompson, C. Downey et al., "The clinical and functional relevance of microparticles induced by activated protein C treatment in sepsis," *Critical Care*, vol. 15, no. 4, article R195, 2011.

[29] A. O. Soriano, W. Jy, J. A. Chirinos et al., "Levels of endothelial and platelet microparticles and their interactions with leukocytes negatively correlate with organ dysfunction and predict mortality in severe sepsis," *Critical Care Medicine*, vol. 33, no. 11, pp. 2540–2546, 2005.

[30] C. Liang, H. Feng, B. Q. Deng et al., "Decreased levels and function of circulating endothelial progenitor cells in unruptured intracranial saccular aneurysm patients," *Neurological Sciences*, vol. 35, no. 1, pp. 23–28, 2013.

[31] L. Liu, H. Liu, J. Jao et al., "Changes in circulating human endothelial progenitor cells after brain injury," *Journal of Neurotrauma*, vol. 24, no. 6, pp. 936–943, 2007.

[32] L. Liu, H. Wei, F. Chen, J. Wang, J.-F. Dong, and J. Zhang, "Endothelial progenitor cells correlate with clinical outcome of traumatic brain injury," *Critical Care Medicine*, vol. 39, no. 7, pp. 1760–1765, 2011.

[33] H.-J. Wei, D. Wang, J.-L. Chen et al., "Mobilization of circulating endothelial progenitor cells after endovascular therapy for ruptured cerebral aneurysms," *Neuroscience Letters*, vol. 498, no. 2, pp. 114–118, 2011.

[34] H. Wei, Q. Mao, L. Liu et al., "Changes and function of circulating endothelial progenitor cells in patients with cerebral aneurysm," *Journal of Neuroscience Research*, vol. 89, no. 11, pp. 1822–1828, 2011.

[35] P. Lackner, A. Dietmann, R. Beer et al., "Cellular microparticles as a marker for cerebral vasospasm in spontaneous subarachnoid hemorrhage," *Stroke*, vol. 41, no. 10, pp. 2353–2357, 2010.

[36] N. Morel, O. Morel, L. Petit et al., "Generation of procoagulant microparticles in cerebrospinal fluid and peripheral blood after traumatic brain injury," *Journal of Trauma—Injury, Infection and Critical Care*, vol. 64, no. 3, pp. 698–704, 2008.

[37] M. R. Sanborn, S. R. Thom, L. E. Bohman et al., "Temporal dynamics of microparticle elevation following subarachnoid hemorrhage," *Journal of Neurosurgery*, vol. 117, no. 3, pp. 579–586, 2012.

[38] L. L. Horstman, W. Jy, J. J. Jimenez, and Y. S. Ahn, "Endothelial microparticles as markers of endothelial dysfunction," *Frontiers in Bioscience*, vol. 9, pp. 1118–1135, 2004.

[39] M. N. Abid Hussein, E. W. Meesters, N. Osmanovic, F. P. H. T. M. Romijn, R. Nieuwland, and A. Sturk, "Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo," *Journal of Thrombosis and Haemostasis*, vol. 1, no. 11, pp. 2434–2443, 2003.

[40] J. J. Jimenez, W. Jy, L. M. Mauro, C. Soderland, L. L. Horstman, and Y. S. Ahn, "Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis," *Thrombosis Research*, vol. 109, no. 4, pp. 175–180, 2003.

[41] W. Jy, L. L. Horstman, J. J. Jimenez et al., "Measuring circulating cell-derived microparticles," *Journal of Thrombosis and Haemostasis*, vol. 2, no. 10, pp. 1842–1851, 2004.

[42] S. H. van Ierssel, E. M. Van Craenenbroeck, V. M. Conraads et al., "Flow cytometric detection of endothelial microparticles (EMP): effects of centrifugation and storage alter with the phenotype studied," *Thrombosis Research*, vol. 125, no. 4, pp. 332–339, 2010.

[43] R. Lacroix, S. Robert, P. Poncelet, R. S. Kasthuri, N. S. Key, and F. Dignat-George, "Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop," *Journal of Thrombosis and Haemostasis*, vol. 8, no. 11, pp. 2571–2574, 2010.

[44] R. Lacroix, C. Judicone, P. Poncelet et al., "Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol," *Journal of Thrombosis and Haemostasis*, vol. 10, no. 3, pp. 437–446, 2012.

[45] S. H. van Ierssel, V. Y. Hoymans, E. M. van Craenenbroeck et al., "Endothelial microparticles (EMP) for the assessment of endothelial function: an *in vitro* and *in vivo* study on possible interference of plasma lipids," *PLoS ONE*, vol. 7, no. 2, Article ID e31496, 2012.

[46] M. S. Goligorsky, "Clinical assessment of endothelial dysfunction: combine and rule," *Current Opinion in Nephrology and Hypertension*, vol. 15, no. 6, pp. 617–624, 2006.

[47] D. A. Harrison, C. A. Welch, and J. M. Eddleston, "The epidemiology of severe sepsis in England, Wales and Northern Ireland, 1996 to 2004: secondary analysis of a high quality clinical database, the ICNARC Case Mix Programme Database," *Critical Care*, vol. 10, no. 2, article R42, 2006.

[48] J.-L. Vincent, Y. Sakr, C. L. Sprung et al., "Sepsis in European intensive care units: results of the SOAP study," *Critical Care Medicine*, vol. 34, no. 2, pp. 344–353, 2006.

[49] R. C. Bone, W. J. Sibbald, and C. L. Sprung, "The ACCP-SCCM consensus conference on sepsis and organ failure," *Chest*, vol. 101, no. 6, pp. 1481–1483, 1992.

[50] J.-L. Vincent, D. R. Nelson, and M. D. Williams, "Is worsening multiple organ failure the cause of death in patients with severe sepsis?" *Critical Care Medicine*, vol. 39, no. 5, pp. 1050–1055, 2011.

[51] J.-L. Vincent, A. De Mendonça, F. Cantraine et al., "Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study," *Critical Care Medicine*, vol. 26, no. 11, pp. 1793–1800, 1998.

[52] M. M. Levy, W. L. Macias, J.-L. Vincent et al., "Early changes in organ function predict eventual survival in severe sepsis," *Critical Care Medicine*, vol. 33, no. 10, pp. 2194–2201, 2005.

[53] O. O. Nduka and J. E. Parrillo, "The pathophysiology of septic shock," *Critical Care Clinics*, vol. 25, no. 4, pp. 677–702, 2009.

[54] N. Matsuda and Y. Hattori, "Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction," *Journal of Smooth Muscle Research*, vol. 43, no. 4, pp. 117–137, 2007.

[55] A. E. Jones and M. A. Puskarich, "Sepsis-induced tissue hypoperfusion," *Critical Care Clinics*, vol. 25, no. 4, pp. 769–779, 2009.

[56] D. de Backer, J. Creteur, J.-C. Preiser, M.-J. Dubois, and J.-L. Vincent, "Microvascular blood flow is altered in patients with sepsis," *American Journal of Respiratory and Critical Care Medicine*, vol. 166, no. 1, pp. 98–104, 2002.

[57] Y. Sakr, M.-J. Dubois, D. De Backer, J. Creteur, and J.-L. Vincent, "Persistent-microcirculatory alterations are associated with organ failure and death in patients with septic shock," *Critical Care Medicine*, vol. 32, no. 9, pp. 1825–1831, 2004.

[58] H. Ait-Oufella, E. Maury, S. Lehoux, B. Guidet, and G. Offenstadt, "The endothelium: physiological functions and role in microcirculatory failure during severe sepsis," *Intensive Care Medicine*, vol. 36, no. 8, pp. 1286–1298, 2010.

[59] M. Mutunga, B. Fulton, R. Bullock et al., "Circulating endothelial cells in patients with septic shock," *American Journal of Respiratory and Critical Care Medicine*, vol. 163, no. 1, pp. 195–200, 2001.

[60] P. Kienbaum, C. Prante, N. Lehmann, A. Sander, A. Jalowy, and J. Peters, "Alterations in forearm vascular reactivity in patients with septic shock," *Anaesthesia*, vol. 63, no. 2, pp. 121–128, 2008.

[61] J. S. Davis, T. W. Yeo, J. H. Thomas et al., "Sepsis-associated microvascular dysfunction measured by peripheral arterial tonometry: an observational study," *Critical Care*, vol. 13, no. 5, article R155, 2009.

[62] L. Becker, K. Prado, M. Foppa et al., "Endothelial dysfunction assessed by brachial artery ultrasound in severe sepsis and septic shock," *Journal of Critical Care*, vol. 27, no. 3, pp. 316.e9–316.e16, 2012.

[63] K. C. Doerschug, A. S. Delsing, G. A. Schmidt, and W. G. Haynes, "Impairments in microvascular reactivity are related to organ failure in human sepsis," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 293, no. 2, pp. H1065–H1071, 2007.

[64] M. E. Astiz, E. Tilly, E. D. Rackow, and M. H. Weil, "Peripheral vascular tone in sepsis," *Chest*, vol. 99, no. 5, pp. 1072–1075, 1991.

[65] C.-F. Lam, Y.-C. Liu, J.-K. Hsu et al., "Autologous transplantation of endothelial progenitor cells attenuates acute lung injury in rabbits," *Anesthesiology*, vol. 108, no. 3, pp. 392–401, 2008.

[66] I. Darwish and W. C. Liles, "Emerging therapeutic strategies to prevent infection-related microvascular endothelial activation and dysfunction," *Virulence*, vol. 4, no. 6, pp. 572–582, 2013.

[67] B. Assmus, C. Urbich, A. Aicher et al., "HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes," *Circulation Research*, vol. 92, no. 9, pp. 1049–1055, 2003.

[68] S. Dimmeler, A. Aicher, M. Vasa et al., "HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway," *Journal of Clinical Investigation*, vol. 108, no. 3, pp. 391–397, 2001.

[69] M. Pérez-Casal, C. Downey, B. Cutillas-Moreno, M. Zuzel, K. Fukudome, and C. H. Ton, "Microparticle-associated endothelial protein C receptor and the induction of cytoprotective and anti-inflammatory effects," *Haematologica*, vol. 94, no. 3, pp. 387–394, 2009.

[70] H.-L. Tian, Z. Geng, Y.-H. Cui et al., "Risk factors for posttraumatic cerebral infarction in patients with moderate or severe head trauma," *Neurosurgical Review*, vol. 31, no. 4, pp. 431–436, 2008.

[71] I. Tawil, D. M. Stein, S. E. Mirvis, and T. M. Scalea, "Posttraumatic cerebral infarction: incidence, outcome, and risk factors," *Journal of Trauma—Injury, Infection and Critical Care*, vol. 64, no. 4, pp. 849–853, 2008.

[72] M. D. I. Vergouwen, D. Ilodigwe, and R. L. MacDonald, "Cerebral infarction after subarachnoid hemorrhage contributes to poor outcome by vasospasm-dependent and -independent effects," *Stroke*, vol. 42, no. 4, pp. 924–929, 2011.

[73] N. K. de Rooij, J. P. Greving, G. J. Rinkel, and C. J. Frijns, "Early prediction of delayed cerebral ischemia after subarachnoid hemorrhage: development and validation of a practical risk chart," *Stroke*, vol. 44, no. 5, pp. 1288–1294, 2013.

[74] R. J. Brown, A. Kumar, R. Dhar, T. R. Sampson, and M. N. Diringer, "The relationship between delayed infarcts and angiographic vasospasm after aneurysmal subarachnoid hemorrhage," *Neurosurgery*, vol. 72, no. 5, pp. 702–708, 2013.

[75] M. D. I. Vergouwen, M. Vermeulen, J. van Gijn et al., "Definition of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage as an outcome event in clinical trials and observational studies: proposal of a multidisciplinary research group," *Stroke*, vol. 41, no. 10, pp. 2391–2395, 2010.

[76] J. P. Dreier, J. Woitzik, M. Fabricius et al., "Delayed ischaemic neurological deficits after subarachnoid haemorrhage are associated with clusters of spreading depolarizations," *Brain*, vol. 129, no. 12, pp. 3224–3237, 2006.

[77] J. M. K. Lam, P. Smielewski, M. Czosnyka, J. D. Pickard, and P. J. Kirkpatrick, "Predicting delayed ischemic deficits after aneurysmal subarachnoid hemorrhage using a transient hyperemic response test of cerebral autoregulation," *Neurosurgery*, vol. 47, no. 4, pp. 819–826, 2000.

[78] M. D. I. Vergouwen, M. Vermeulen, B. A. Coert, E. S. G. Stroes, and Y. B. W. E. M. Roos, "Microthrombosis after aneurysmal subarachnoid hemorrhage: an additional explanation for delayed cerebral ischemia," *Journal of Cerebral Blood Flow and Metabolism*, vol. 28, no. 11, pp. 1761–1770, 2008.

[79] M. Sabri, J. Ai, K. Lakovic, J. D'Abbondanza, D. Ilodigwe, and R. L. Macdonald, "Mechanisms of microthrombi formation after experimental subarachnoid hemorrhage," *Neuroscience*, vol. 224, pp. 26–37, 2012.

[80] G. W. Britz, J. R. Meno, I.-S. Park et al., "Time-dependent alterations in functional and pharmacological arteriolar reactivity after subarachnoid hemorrhage," *Stroke*, vol. 38, no. 4, pp. 1329–1335, 2007.

[81] E. Kóniewska, R. Michalik, J. Rafałowska et al., "Mechanisms of vascular dysfunction after subarachnoid hemorrhage," *Journal of Physiology and Pharmacology*, vol. 57, no. 11, pp. 145–160, 2006.

[82] K. W. Park, C. Metais, H. B. Dai, M. E. Comunale, and F. W. Sellke, "Microvascular endothelial dysfunction and its mechanism in a rat model of subarachnoid hemorrhage," *Anesthesia and Analgesia*, vol. 92, no. 4, pp. 990–996, 2001.

[83] S. Park, M. Yamaguchi, C. Zhou, J. W. Calvert, J. Tang, and J. H. Zhang, "Neurovascular protection reduces early brain injury after subarachnoid hemorrhage," *Stroke*, vol. 35, no. 10, pp. 2412–2417, 2004.

[84] Y. Kubo, K. Ogasawara, S. Kakino et al., "Serum inflammatory adhesion molecules and high-sensitivity C-reactive protein correlates with delayed ischemic neurologic deficits after subarachnoid hemorrhage," *Surgical Neurology*, vol. 69, no. 6, pp. 592–596, 2008.

[85] A. Server, R. Dullerud, M. Haakonsen, P. H. Nakstad, U. L.-H. Johnsen, and B. Magnæs, "Post-traumatic cerebral infarction: neuroimaging findings, etiology and outcome," *Acta Radiologica*, vol. 42, no. 3, pp. 254–260, 2001.

[86] S. S. Armin, A. R. T. Colohan, and J. H. Zhang, "Vasospasm in traumatic brain injury," *Acta Neurochirurgica, Supplementum*, vol. 104, no. 13, pp. 421–425, 2008.

[87] M. Taneda, K. Kataoka, F. Akai, T. Asai, and I. Sakata, "Traumatic subarachnoid hemorrhage as a predictable indicator of delayed ischemic symptoms," *Journal of Neurosurgery*, vol. 84, no. 5, pp. 762–768, 1996.

[88] E. Park, J. D. Bell, I. P. Siddiq, and A. J. Baker, "An analysis of regional microvascular loss and recovery following two grades of fluid percussion trauma: a role for hypoxia-inducible factors in traumatic brain injury," *Journal of Cerebral Blood Flow and Metabolism*, vol. 29, no. 3, pp. 575–584, 2009.

[89] H. A. Kontos and E. P. Wei, "Endothelium-dependent responses after experimental brain injury," *Journal of Neurotrauma*, vol. 9, no. 4, pp. 349–354, 1992.

[90] M. D. I. Vergouwen, K. Bakhtiari, N. Van Geloven, M. Vermeulen, Y. B. Roos, and J. C. M. Meijers, "Reduced ADAMTS13 activity in delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage," *Journal of Cerebral Blood Flow and Metabolism*, vol. 29, no. 10, pp. 1734–1741, 2009.

[91] C. J. M. Frijns, R. Fijnheer, A. Algra, J. A. van Mourik, J. van Gijn, and G. J. E. Rinkel, "Early circulating levels of endothelial cell activation markers in aneurysmal subarachnoid haemorrhage: associations with cerebral ischaemic events and outcome," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 77, no. 1, pp. 77–83, 2006.

[92] J. J. Nissen, D. Mantle, B. Gregson, and A. D. Mendelow, "Serum concentration of adhesion molecules in patients with delayed ischaemic neurological deficit after aneurysmal subarachnoid haemorrhage: the immunoglobulin and selectin superfamilies," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 71, no. 3, pp. 329–333, 2001.

[93] B. Wang, L. Sun, Y. Tian et al., "Effects of atorvastatin in the regulation of circulating EPCs and angiogenesis in traumatic brain injury in rats," *Neurological Sciences*, vol. 319, no. 1-2, pp. 117–123, 2012.

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Research Article

Short-Term Esmolol Improves Coronary Artery Remodeling in Spontaneously Hypertensive Rats through Increased Nitric Oxide Bioavailability and Superoxide Dismutase Activity

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The aim of this study was to assess the effects of short-term esmolol therapy on coronary artery structure and function and plasma oxidative stress in spontaneously hypertensive rats (SHR). For this purpose, 14-month-old male SHR were treated for 48 hours with esmolol (SHR-E, 300 μg/kg/min). Age-matched untreated male SHR and Wistar Kyoto rats (WKY) were used as hypertensive and normotensive controls, respectively. At the end of intervention we performed a histological study to analyze coronary artery wall width (WW), wall-to-lumen ratio (W/L), and media cross-sectional area (MCSA). Dose-response curves for acetylcholine (ACh) and sodium nitroprusside were constructed. We also assessed several plasma oxidative stress biomarkers, namely, superoxide scavenging activity (SOSA), nitrites, and total antioxidant capacity (TAC). We observed a significant reduction in WW ($P < 0.001$), W/L ($P < 0.05$), and MCSA ($P < 0.01$) and improved endothelium-dependent relaxation ($AUC_{SHR\text{-}E} = 201.2 \pm 33$ versus $AUC_{SHR} = 97.5 \pm 21$, $P < 0.05$) in SHR-E compared with untreated SHR; no differences were observed for WW, MCSA, and endothelium-dependent relaxation by ACh at higher concentrations (10^{-6} to 10^{-4} mol/l) for SHR-E with respect to WKY. SOSA ($P < 0.001$) and nitrite ($P < 0.01$) values were significantly higher in SHR-E than in untreated SHR; however, TAC did not increase after treatment with esmolol. Esmolol improves early coronary artery remodeling in SHR.

1. Introduction

The risk of fatal cardiovascular events is associated with adverse structural and functional remodeling of the vasculature, which is frequently caused by hypertension [1]. Regression of these changes is a goal of antihypertensive therapy [2] and is associated with reduced incidence of cardiovascular events [3, 4]. Chronic treatment with antihypertensive agents (angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, calcium antagonists, β-blockers, and diuretics) has led to regression of vascular remodeling both in humans and in animal models of hypertension [2, 5, 6]. However, early regression of coronary artery remodeling after short-term therapy with these agents has not been investigated.

Esmolol is an ultrashort-acting cardioselective β-adrenergic blocker with a half-life of ~2 minutes, a time to peak effect of about 6–10 minutes, and a washout time of 9 minutes [7]. Therefore, esmolol is faster acting than other β-blockers and is an excellent option for the treatment of

arterial hypertension and undesirable increases in heart rate [8], although its effect on vascular remodeling has not been established.

Our group previously demonstrated that short-term treatment (48 hours) with esmolol reduces left ventricular hypertrophy in spontaneously hypertensive rats (SHR) [9]. However, early changes in vascular remodeling and oxidative stress following short-term use of this agent have not been analyzed to date.

In the present study, we hypothesized that even short-term administration (48 hours) of esmolol could reduce structural and functional coronary artery remodeling by increasing the bioavailability of nitric oxide (NO) and superoxide dismutase activity (SOD) in SHR. Therefore, we analyzed the protective effect of esmolol in coronary arteries and antioxidant status in plasma.

2. Methods

All procedures conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23, revised in 1996) and Spanish legislation (RD 1201/2005) and were approved by the Ethics Committee of Hospital General Universitario Gregorio Marañon, Madrid, Spain.

2.1. Experimental Design. The study animals—14-month-old male SHR ($n = 22$) and normotensive control Wistar-Kyoto (WKY) rats ($n = 11$)—were bred at the animal house of Universidad Autónoma de Madrid. All the rats were supplied with standard rat chow and drinking water ad libitum and were maintained on a 12 h/12 h light/dark cycle. The animals were housed at a constant temperature of 24°C and relative humidity of 40%. All the rats were anesthetized with an intraperitoneal injection of diazepam 4 mg/kg/1 and ketamine 10 mg/kg/1, and a catheter was inserted into the right internal jugular vein under sterile conditions. SHR were randomly divided into 2 groups (11 rats each): rats treated with esmolol (SHR-E) and rats treated with vehicle (SHR, hypertensive control group). SHR-E received an intravenous infusion of esmolol at 300 μg/kg/min for 48 hours. SHR and WKY received saline solution (vehicle). After 48 hours of treatment, the rats were killed by decapitation. Blood samples and the heart were removed immediately to study oxidative stress and perform structural and vascular reactivity experiments.

2.2. Blood Pressure and Heart Rate Measurements. Systolic arterial pressure (SAP) and heart rate (HR) were measured (conscious animals prewarmed to 35°C in thermostatic cages) by the tail-cuff method with a photoelectric sensor (Niprem 546, Cibertec, Madrid, Spain). Several determinations were made and the findings were considered valid if 10 consecutive measurements were within 10 mmHg of each other.

2.3. Morphology of Intramyocardial Arteries. We analyzed 5 animals from each group. Left ventricular tissue was fixed in 4% sodium-buffered formaldehyde. We made sequential transversal cuts of the left ventricle from the subvalvular region to the apex. Samples were then dehydrated and embedded in paraffin. Serial sections (5 μm) were stained with orcein. We differentiated between the subepicardial and midmyocardial parts of the left ventricular wall, following the procedure described by Lunkenheimer et al. [10]. We then located the intramyocardial branch of the obtuse marginal artery (branch of the circumflex coronary artery). A total of 30 intramyocardial arteries (2 vessels per rat) were observed and analyzed using a high-resolution camera (Sony CCD IRIS) attached to a microscope (Leica DMLB, ×40). The morphometric analyses were performed using the method of Gundersen el al. [11]. The images were projected on a computer screen, and the external diameter (ED) (inner diameter + tunica intima + tunica media) and lumen diameter (LD) of the coronary arteries were measured. The wall width (WW) was expressed as (ED-LD)/2. The wall-to-lumen ratio (W/L) was expressed as (WW/LD) × 100, and the media cross-sectional area (MCSA) (tunica intima + tunica media) was expressed as $(\pi/4) \times (ED^2 - LD^2)$ [6].

2.4. Blood Collection and Plasma Preparation. We analyzed 6 animals from each group. Blood (2.4 mL) was collected from each animal in Vacutainer tubes (BD, Plymouth, UK) containing citrate (300 μL). Blood samples were centrifuged at 900 g for 10 minutes at 4°C to obtain plasma, which was aliquoted and stored at −80°C for further analysis.

2.4.1. Nitrite. The plasma nitrite level was assessed using a Griess reaction-based protocol adapted from Giustarini et al. [12] and Miranda et al. [13]. One hundred microliters of plasma or nitrite standard (0–100 μM) was mixed with 10 μL de N-ethylenediamine (150 mM) to eliminate thiols that interfere with the Griess reaction. Thereafter, the protein from samples was precipitated by addition of 110 μL of trichloroacetic acid (20% w/v) and incubation in an ice bath for 5 minutes. Fifty microliters of supernatant obtained by centrifugation at 12,000 g for 5 minutes (4°C) was mixed on 96-well plate with 50 μL of saturated vanadium chloride (dissolved in HCl 1 M), 25 μL of sulphanilamide (2% w/v in HCl 5% v/v), and 25 μL of N-naphthyl-ethylenediamine (0.1% w/v in water). Finally, the mixture was incubated at 37°C for 1 hour, and the absorbance was read at 540 nm. Plasma nitrite content was expressed in μM.

2.4.2. Quantification of Superoxide Scavenging Activity (SOSA). Plasma superoxide anion scavenging activity was assessed using the SOSA assay based on the inhibition of luminescence emitted by coelenterazine, which is oxidized by superoxide anion [14]. Superoxide anion was produced using a hypoxanthine/xanthine oxidase system. SOSA values were quantified by comparing the luminescence inhibition of each sample with that observed on the superoxide dismutase activity standard curve (0–4 mU/mL) and expressed as mU/mL.

2.4.3. Catalase Activity. Catalase activity was assessed using the Amplex red catalase assay (Catalase Assay Kit with

Amplex Ultra Red reagent; Invitrogen). Catalase activity was expressed as units per milligram of protein.

2.4.4. Total Antioxidant Capacity (TAC). TAC was assessed using a modified CUPRAC-BCS assay. Briefly, 10 μL of diluted plasma with phosphate buffer (10 mM, pH = 7.4) was mixed on 96-well plate with 190 μL of BCS 0.25 mM (dissolved in phosphate buffer), and the initial absorbance (Ai) was measured at 490 nm. Thereafter, 50 μL of $CuSO_4$ (0.5 mM dissolved in water) was added, the mix was incubated in darkness at room temperature for 5 minutes, and the final absorbance (Af) was measured at 490 nm. TAC was calculated from (Af-Ai) and the standard curve and expressed as mM Trolox.

2.4.5. Total Thiols. Plasma thiols were assessed using the microplate 5,5′-dithiobis (2-nitrobenzoic acid) assay [15]. The absorbance was measured at 412 nm in a Synergy HT Multi-Mode Microplate Reader (BioTek, Potton, UK), and thiol content was expressed as millimoles per liter of reduced glutathione per milligram of protein.

2.4.6. Protein Content. Protein content was assessed using a Coomassie blue-based microtiter plate assay with bovine serum albumin as standard (Bio-Rad). The absorbance was measured at 595 nm in a Synergy HT Multi-Mode Microplate Reader.

2.5. Vascular Reactivity of Coronary Arteries. We analyzed 6 animals from each group. The heart was removed and maintained in cold (4°C) oxygenated Krebs-Henseleit solution (KHS, in mmol·L^{-1}: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose, and 0.01 Na_2EDTA). Segments of the left anterior descending artery were isolated, and surrounding cardiac tissue was cleaned under a dissecting microscope. Segments of coronary arteries (2 mm in length) were mounted on a wire myograph (Multi Myograph System, model 610 M; Danish Myo-Technology) coupled to a Powerlab data acquisition system (AD-Instruments, Castle Hill, Australia) and studied as described elsewhere [16]. Briefly, the vessel was mounted in oxygenated KHS. The arteries were then set to a normalized internal circumference of 0.9 L100, which was considered as the effective lumen diameter that represented the basal tone of the artery. After an equilibration period in KHS at 37°C and pH 7.4, segments were stretched to their optimal lumen diameter to develop active tension. Coronary arteries were exposed to 120 mmol·L^{-1} K^+-KHS in order to ensure their functional integrity. Responses to acetylcholine (ACh, 10^{-9} to 10^{-4} mol/l) were studied in segments precontracted with 5-hydroxytryptamine (5-HT, 3×10^{-7} mol/l). After a washout period of 60 minutes, dose-response curves were constructed for sodium nitroprusside (SNP, 10^{-9} to 10^{-4} mol/l) to study endothelium-independent relaxation in segments precontracted with 5-HT (3×10^{-7} mol/l).

2.6. Data Analysis and Statistics. The results were expressed as mean ± SEM. Between-group comparisons were made by 1-way analysis of variance followed by a Bonferroni post hoc test. Relaxing responses are expressed as percentage reduction in the 5-HT preconstricted state. To compare the effect of vasodilator drugs on response to 5-HT in coronary segments, some results were expressed as differences in the area under the concentration-response curves between the 3 experimental groups. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using SPSS 17.0 for windows (SPSS, Chicago, Illinois, USA) and S-PLUS 6.1.

3. Results

3.1. Physiological Parameters. Body weight was higher in WKY than in SHR (441.60 ± 22.08 g versus 405.13 ± 22.12 g, $P < 0.05$) and SHR-E (441.60±22.08 g versus 400.15±11.07 g, $P < 0.05$). Heart weight was higher in SHR than in WKY (2.10±0.27 g versus 1.51±0.11 g, $P < 0.05$) but decreased after treatment in SHR-E compared with SHR (1.49 ± 0.12 g versus 2.10 ± 0.27 g, $P < 0.05$). There were no significant differences in heart weigh between SHR-E and WKY.

3.2. Hemodynamic Parameters. SAP was higher in SHR than in WKY (236 ± 1.5 versus 135 ± 0.1, $P < 0.001$). After 48 hours of treatment, esmolol lowered SAP in SHR-E with respect to SHR (149 ± 2 versus 236 ± 1.5, $P < 0.001$), and the SAP values at the end of treatment were not comparable with those of the WKY ($P < 0.05$). Heart rate remained unchanged in both SHR and WKY (297 ± 1 versus 297 ± 2) but decreased after treatment in SHR-E compared with SHR (183 ± 3 versus 297 ± 1, $P < 0.001$) and WKY ($P < 0.001$).

3.3. Effect of Esmolol on Intramyocardial Artery Morphology. Intramyocardial artery ED was significantly greater in SHR than in WKY. Administration of esmolol to SHR-E decreased ED, although the difference was not statistically significant compared with untreated SHR (Figures 1(a) and 2). The LD of the artery in SHR was significantly greater than in WKY. Administration of esmolol increased LD by a greater amount in SHR-E than in SHR, although the difference was not statistically significant (Figures 1(b) and 2). The WW of the artery was significantly higher in SHR than in WKY. Interestingly, WW was significantly lower after 48 hours of treatment in SHR-E than in SHR, although no differences were detected with respect to WKY (Figures 1(c) and 2). The W/L in SHR did not differ from that in the WKY. Administration of esmolol significantly decreased the W/L ratio in SHR-E (Figures 1(d) and 2). The MCSA in the SHR was larger than in the WKY. Interestingly, MCSA was significantly lower after 48 hours of treatment in SHR-E than in SHR; no differences were observed with respect to WKY (Figures 1(e) and 2).

3.4. Effect of Esmolol on Coronary Artery Vasodilator Function. In WKY and SHR-E rats, ACh elicited concentration-dependent relaxation at all the concentrations tested, whereas in SHR, ACh elicited a biphasic response with dilatation at low concentrations (10^{-9} to 10^{-5} mol/l) and contractions

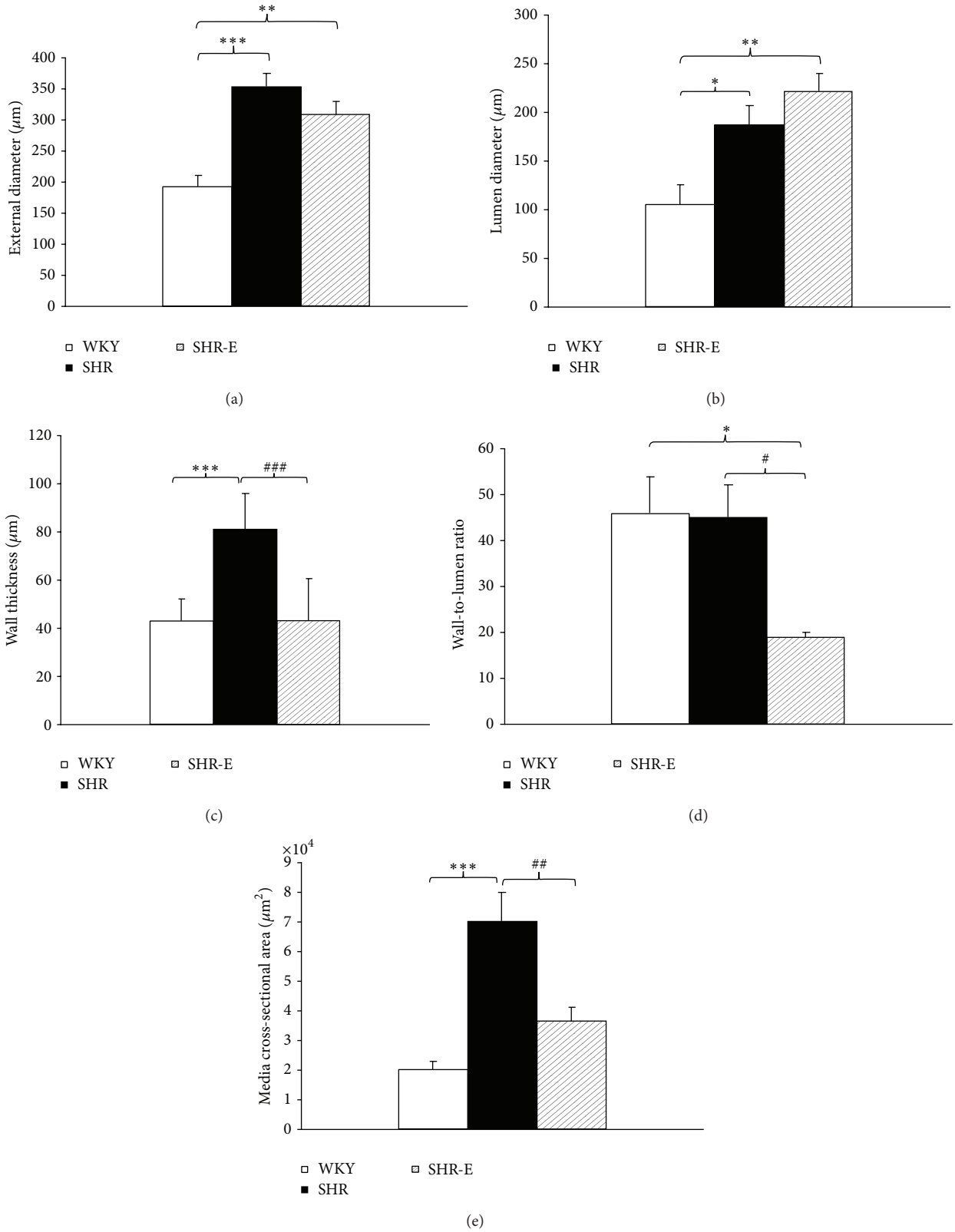

Figure 1: Structural parameters. (a) External diameter, (b) lumen diameter, (c) wall thickness, (d) wall-to-lumen ratio, and (e) media cross-sectional area of intramyocardial artery of the left ventricle from Wistar Kyoto control rats (WKY, $n = 5$), spontaneously hypertensive control rats (SHR, $n = 5$), and spontaneously hypertensive rats treated with esmolol (SHR-E, $n = 5$). Data are expressed as mean ±SEM. Statistically significant differences between WKY, SHR, and SHR-E are shown ($^{*}P < 0.05$ versus WKY; $^{**}P < 0.01$ versus WKY; $^{***}P < 0.001$ versus WKY; $^{\#}P < 0.05$ versus SHR; $^{\#\#}P < 0.01$ versus SHR; $^{\#\#\#}P < 0.001$ versus SHR).

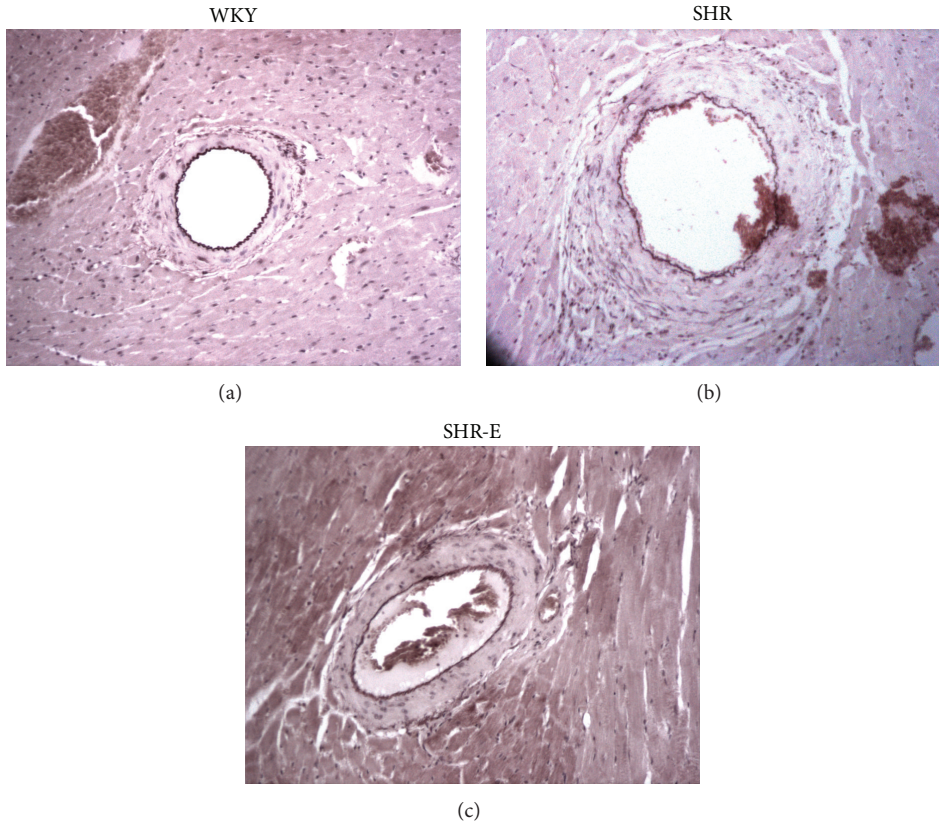

Figure 2: Examples of histological sections of the intramyocardial artery of the left ventricle from a Wistar Kyoto control rat (WKY), spontaneously hypertensive control rat (SHR), and spontaneously hypertensive rat treated with esmolol (SHR-E). Orcein x10.

at higher concentrations (10^{-4} mol/l). The endothelium-dependent relaxation induced by ACh in 5-HT contracted coronary arteries was significantly lower in SHR than in WKY (10^{-7} to 10^{-4} mol/l), and esmolol significantly improved this relaxation (10^{-6} to 10^{-4}). Treatment with esmolol normalized coronary artery endothelium-dependent relaxation by ACh at higher concentrations (10^{-6} to 10^{-4} mol/l) (Figure 3(a)). The AUC was significantly larger in WKY than in SHR (AUC_{WKY} = 265.9 ± 27 versus AUC_{SHR} = 97.5 ± 21, P = 0.0002). AUC was significantly higher in SHR-E than in SHR ($AUC_{SHR\text{-}E}$ = 201.2 ± 33 versus AUC_{SHR} = 97.5 ± 21, P = 0.027); no differences were observed for SHR-E with respect to WKY.

No differences in the concentration-response curves for the endothelium-independent vasodilator SNP (10^{-9} to 10^{-4} mol/l) were observed between the 3 experimental groups (AUC_{WKY} = 292.9 ± 34; AUC_{SHR} = 253.2 ± 19; $AUC_{SHR\text{-}E}$ = 307.2 ± 34) (Figure 3(b)). Maximal dilatation to SNP was 97.7 ± 1% in WKY, 91.4 ± 2% in SHR, and 98.6 ± 1% in SHR-E (Figure 3(b)).

3.5. Effect of Esmolol on NO Level and Plasma Antioxidant Status. Interestingly, plasma nitrite level was significantly higher after 48 hours of treatment in SHR-E than in SHR (Figure 4(a)). No differences were observed between SHR and WKY, although a trend toward reduction was recorded (Figure 4(a)). Moreover, the SOSA value, which is in part related to superoxide dismutase activity, was significantly increased after treatment with esmolol in SHR-E, although no differences were observed for this enzyme in SHR compared with WKY (Figure 4(b)). Similarly, administration of esmolol increased catalase activity in SHR-E but not in SHR or WKY (Figure 4(c)). Finally, administration of esmolol did not change total plasma antioxidant activity (Figure 4(d)) or thiol level (Figure 4(e)) in SHR-E, SHR, or WKY.

4. Discussion

Our results show that short-term (48 hours) intravenous infusion of esmolol (300 μg/kg/min) in adult SHR induces early changes of coronary artery remodeling by increased bioavailability of NO and improved antioxidant status in plasma. This is the first study to show an association between early improvement in coronary artery remodeling with short-term administration of a β-blocker. Although previous human and experimental animal studies have demonstrated that various antihypertensive drugs can reverse coronary artery remodeling, they have all been conducted with drugs administered over several months [17–22].

4.1. Effect of Esmolol on Vascular Structure and Function. Hypertension is associated with structural and functional

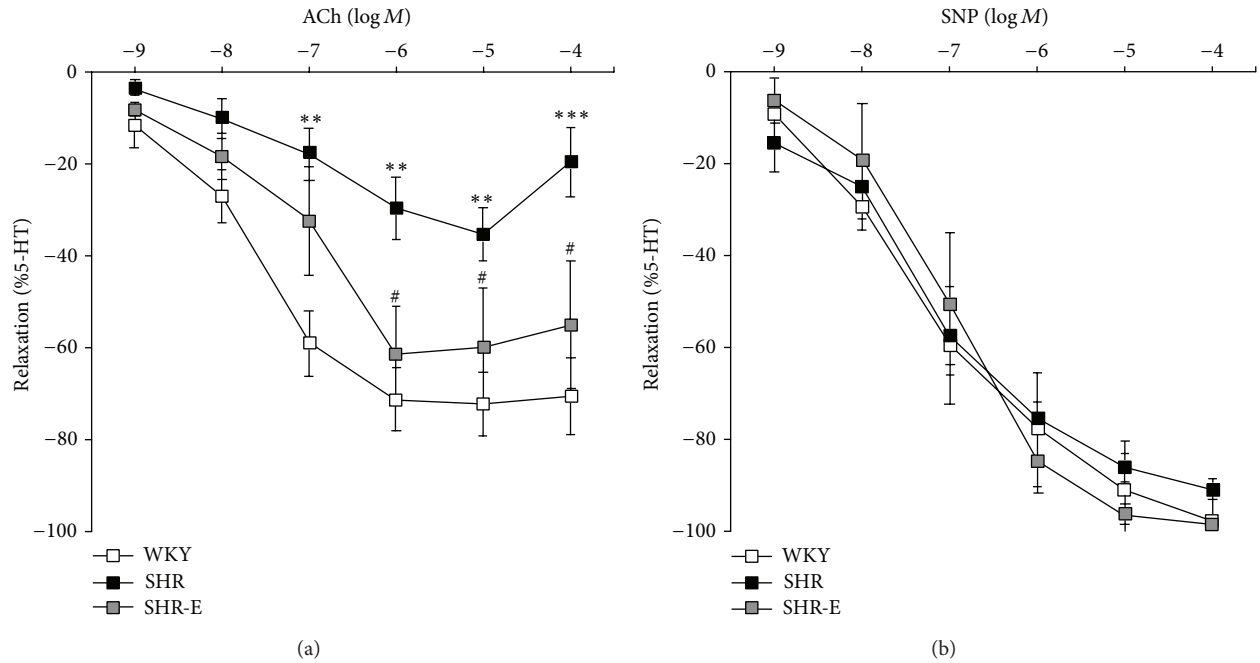

Figure 3: Vascular reactivity parameters. (a) acetylcholine (ACh) responses and (b) sodium nitroprusside (SNP) in left anterior descending artery precontracted with 5-hydroxytryptamine (5-HT, 3×10^{-7} mol/l) from Wistar Kyoto control rats (WKY, $n = 6$), spontaneously hypertensive control rats (SHR, $n = 6$), and spontaneously hypertensive rat treated with esmolol (SHR-E, $n = 6$). Relaxing responses are expressed as percentage reduction in the 5-HT preconstricted state. Data are expressed as mean ± SEM. Statistically significant differences between WKY, SHR, and SHR-E are shown ($^{**}P < 0.01$ versus WKY; $^{***}P < 0.001$ versus WKY; $^{\#}P < 0.05$ versus SHR).

changes in vascular remodeling, which in turn increase cardiovascular risk [23]. In our experiments, we found that WW, MCSA, and LD of intramyocardial arteries in 14-month-old SHR were higher than in WKY. These data are consistent with those of Cebova and Kristek [24], who reported remodeling in the septal branch of the descending coronary artery in 13-month-old SHR; however, the authors found no differences in young SHR and age-matched WKY. The gradual increase in all 3 parameters (WW, MCSA, and LD) with age in coronary artery hypertrophy in SHR could reflect an adaptive mechanism triggered by chronic arterial hypertension.

At 14 months of age, SHR present compensated left ventricular hypertrophy (LVH) [25], which is associated with functional and structural alterations of the coronary artery [26]. We previously reported that 48-hour treatment of SHR with esmolol produces regression of LVH [9], and the results of the present study suggest that coronary artery WW and MCSA in SHR normalizes to those of WKY after treatment. These data are consistent with the effect of other antihypertensive agents (losartan [17], perindopril [18], amlodipine and enalapril [19], indapamide [20], and carvedilol [22]) in coronary artery remodeling, although only after long-term treatment. Regression of vascular remodeling is difficult, even with long-term pharmacological treatment [27], although esmolol produced changes in vascular structure and function in 48 hours. This effect may be attributed in part to the fact that short-term intravenous administration of esmolol exerts a greater hypotensive effect than other drugs [28]. We do not know whether esmolol plays a role in fibrosis, hypertrophy, or both in the coronary arteries. However, in a previous study [9], we showed that esmolol (300 μg/kg/min for 48 h) produces early changes in cross-sectional area of left ventricular cardiomyocytes (in subepicardial region) in SHR but does not affect collagen volume fraction.

Our results show hypertrophic outward remodeling (associated with an increase in arterial wall mass and lumen thickness) [1] in intramyocardial arteries in 14-month-old SHR. However, esmolol produced eutrophic outward remodeling (decreases in wall thickness to inner diameter ratio), which is associated with a decrease in vascular resistance and, consequently, a decrease in arterial pressure [29].

4.2. Effect of Esmolol on Plasma Antioxidant Parameters. Esmolol is an ultrashort-acting specific blocker of β1-adrenergic receptors with an elimination half-life of 9 minutes [30]; in other words, recovery from β blockade (i.e., heart rate approaching baseline levels) can be achieved within 10 minutes of discontinuing the infusion [31]. We recently demonstrated that short-term treatment with esmolol can reverse early left ventricular hypertrophy in the SHR model of stable compensated ventricular hypertrophy [9], although the underlying mechanism has not been explored. The level of plasma nitrite in SHR-E was significantly higher than in the SHR and WKY groups. Nevertheless, a similar improvement has been reported for other blockers of β-adrenergic receptors, such as nebivolol [32], carvedilol [33], and nipradilol [34]. The increase in plasma NO level after administration of esmolol to SHR was markedly greater than with β1-adrenergic

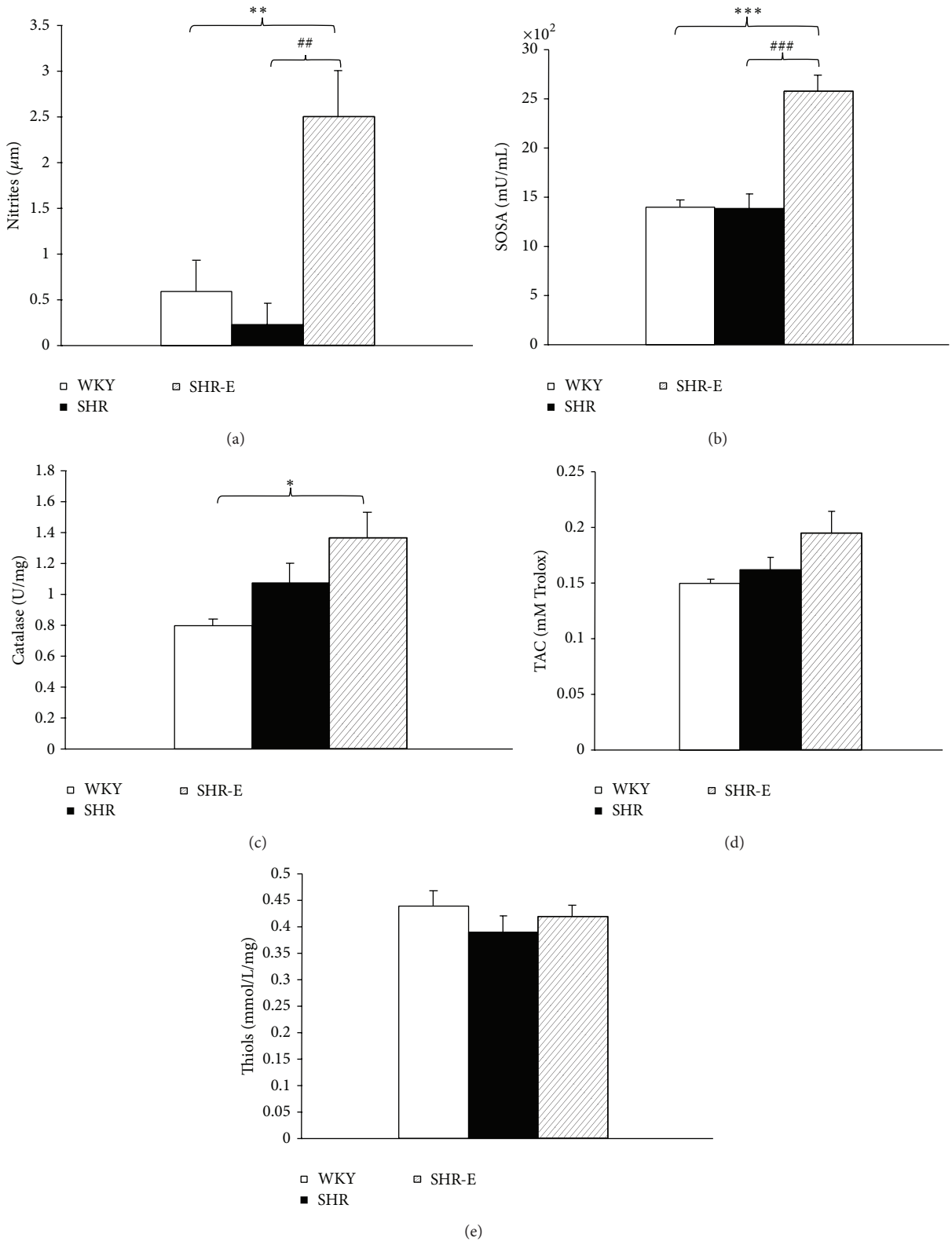

FIGURE 4: Antioxidant parameters. (a) Nitrites, (b) superoxide scavenging activity (SOSA), (c) catalase, (d) total antioxidant capacity (TAC), and (e) thiols on plasma from Wistar Kyoto control rats (WKY, $n = 6$), spontaneously hypertensive control rats (SHR, $n = 6$), and spontaneously hypertensive rat treated with esmolol (SHR-E, $n = 6$). Data are expressed as mean ± SEM. Statistically significant differences between WKY, SHR, and SHR-E are shown ($^{*}P < 0.05$ versus WKY; $^{**}P < 0.01$ versus WKY; $^{***}P < 0.001$ versus WKY; $^{\#\#}P < 0.01$ versus SHR; $^{\#\#\#}P < 0.001$ versus SHR).

receptor blockers, even in WKY [32]. On the other hand, the reduced asymmetric dimethylarginine (ADMA) concentration in SHR treated with nebivolol, a third-generation β-blocker, has been associated with endothelial NO synthase (eNOS) agonist properties and increased bioavailability of NO [32]. In addition, ADMA has been shown to increase ROS level in arterioles in vitro by activation of the vascular renin-angiotensin system [35]. In our study, we showed that SOSA, which includes low-molecular weight antioxidant (scavengers) and superoxide dismutase, increased as a result of treatment with esmolol. The fact that total antioxidant activity does not increase after treatment with esmolol reflects an increase in superoxide dismutase activity measured using the SOSA assay. Likewise, catalase activity was also increased by esmolol in SHR. Esmolol does not exert its antioxidant activity through ROS-scavenging mechanisms [33] similar to those found in this study, and total antioxidant activity did not change with esmolol, although plasma superoxide dismutase activity and catalase activity can be induced by esmolol. This explains how the increase in plasma NO level in SHR after treatment with esmolol was markedly greater than with other β-blockers. Based on these results, esmolol can improve the oxidative stress associated with arterial hypertension. Antioxidant capacity is one of the mechanisms that underlie the efficacy of antihypertensive therapy [36].

Esmolol increases nitric oxide bioavailability and superoxide dismutase activity. These effects could explain structural changes and attenuation of coronary artery dysfunction. However, the vascular remodeling (structural changes) in hypertension is also associated with activation of the renin-angiotensin system, endothelin-1, endothelial dysfunction, oxidative stress, and ADMA [37].

4.3. Study Limitations. This study was designed to analyze the effect of esmolol on the structure and function of the coronary arteries. Although esmolol produces changes in coronary artery structure and function, we cannot conclude that regression of vascular remodeling occurred, because we did not study aspects such as markers of smooth muscle proliferation, activity of matrix metalloproteinases, and staining of perivascular fibrosis. Future studies will be necessary to answer this question.

In conclusion, our results show that esmolol improves coronary artery remodeling by increasing bioavailability of NO and improving antioxidant status in plasma in SHR, because lower WW and lower MCSA, improved endothelial dysfunction, and increased plasma NO level and superoxide dismutase activity were observed in SHR-E compared with the control SHR. Ours is the first study to show improvement of coronary artery remodeling with a β-blocker in the short term. If these results are confirmed in humans, esmolol could be taken into consideration for the treatment of patients with coronary artery disorders caused by arterial hypertension.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] M. Galderisi and O. de Divitiis, "Risk factor-induced cardiovascular remodeling and the effects of angiotensin-converting enzyme inhibitors," *Journal of Cardiovascular Pharmacology*, vol. 51, no. 6, pp. 523–531, 2008.

[2] E. Agabiti-Rosei and D. Rizzoni, "Regression of small resistance artery structural alterations in hypertension by appropriate antihypertensive treatment," *Current Hypertension Reports*, vol. 12, no. 2, pp. 80–85, 2010.

[3] A. Rehman and E. L. Schiffrin, "Vascular effects of antihypertensive drug therapy," *Current Hypertension Reports*, vol. 12, no. 4, pp. 226–232, 2010.

[4] E. L. Schiffrin, "Vascular remodeling and endothelial function in hypertensive patients: effects of antihypertensive therapy," *Scandinavian Cardiovascular Journal, Supplement*, vol. 32, no. 47, pp. 15–21, 1998.

[5] D. Rizzoni and E. Agabiti-Rosei, "Structural abnormalities of small resistance arteries in essential hypertension," *Internal and Emergency Medicine*, vol. 7, no. 3, pp. 205–212, 2011.

[6] H. Yokoyama, D. B. Averill, K. B. Brosnihan, R. D. Smith, E. L. Schiffrin, and C. M. Ferrario, "Role of blood pressure reduction in prevention of cardiac and vascular hypertrophy," *American Journal of Hypertension*, vol. 18, no. 7, pp. 922–929, 2005.

[7] G. Fita, C. Gomar, and I. Rovira, "Esmolol in anesthesiology: pharmacology and indications," *Revista Española de Anestesiología y Reanimación*, vol. 46, no. 9, pp. 401–414, 1999.

[8] D. B. Wiest and J. S. Haney, "Clinical pharmacokinetics and therapeutic efficacy of esmolol," *Clinical Pharmacokinetics*, vol. 51, no. 6, pp. 347–356, 2012.

[9] B. Quintana-Villamandos, M. J. Delgado-Martos, J. J. Sanchez-Hernandez et al., "Early regression of left ventricular hypertrophy after treatment with esmolol in an experimental rat model of primary hypertension," *Hypertension Research*, vol. 36, no. 5, pp. 408–413, 2013.

[10] P. P. Lunkenheimer, K. Redmann, N. Kling et al., "Three-dimensional architecture of the left ventricular myocardium," *The Anatomical Record A, Discoveries in Molecular, Cellular, and Evolutionary Biology*, vol. 288, no. 6, pp. 565–578, 2006.

[11] H. J. G. Gundersen, T. F. Bendtsen, L. Korbo et al., "Some new, simple and efficient stereological methods and their use in pathological research and diagnosis," *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, vol. 96, no. 5, pp. 379–394, 1988.

[12] D. Giustarini, R. Rossi, A. Milzani, and I. Dalle-Donne, "Nitrite and nitrate measurement by griess reagent in human plasma: evaluation of interferences and standardization," *Methods in Enzymology*, vol. 440, pp. 361–380, 2008.

[13] K. M. Miranda, M. G. Espey, and D. A. Wink, "A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite," *Nitric Oxide—Biology and Chemistry*, vol. 5, no. 1, pp. 62–71, 2001.

[14] L. Saleh and C. Plieth, "A coelenterazine-based luminescence assay to quantify high-molecular-weight superoxide anion scavenger activities," *Nature Protocols*, vol. 5, no. 10, pp. 1635–1641, 2010.

[15] C. L. Hawkins, P. E. Morgan, and M. J. Davies, "Quantification of protein modification by oxidants," *Free Radical Biology and Medicine*, vol. 46, no. 8, pp. 965–988, 2009.

[16] M. V. Conde, J. Marín, and G. Balfagón, "Superoxide anion and K^+ channels mediate electrical stimulation-induced relaxation in the rat basilar artery," *European Journal of Pharmacology*, vol. 372, no. 2, pp. 179–186, 1999.

[17] R. Koprdová, M. Cebová, and F. Kristek, "Long-term effect of losartan administration on blood pressure, heart and structure of coronary artery of young spontaneously hypertensive rats," *Physiological Research*, vol. 58, no. 3, pp. 327–335, 2009.

[18] N. K. Thybo, N. Korsgaard, S. Eriksen, K. L. Christensen, and M. J. Mulvany, "Dose-dependent effects of perindopril on blood pressure and small-artery structure," *Hypertension*, vol. 23, no. 5, pp. 659–666, 1994.

[19] A. M. Sharifi, J.-S. Li, D. Endemann, and E. L. Schiffrin, "Effects of enalapril and amlodipine on small-artery structure and composition, and on endothelial dysfunction in spontaneously hypertensive rats," *Journal of Hypertension*, vol. 16, no. 4, pp. 457–466, 1998.

[20] D. Neglia, E. Fommei, A. Varela-Carver et al., "Perindopril and indapamide reverse coronary microvascular remodelling and improve flow in arterial hypertension," *Journal of Hypertension*, vol. 29, no. 2, pp. 364–372, 2011.

[21] J.-S. Li, A. M. Sharifi, and E. L. Schiffrin, "Effect of AT1 angiotensin-receptor blockade on structure and function of small arteries in SHR," *Journal of Cardiovascular Pharmacology*, vol. 30, no. 1, pp. 75–83, 1997.

[22] F. C. Barone, W. G. Campbell Jr., A. H. Nelson, and G. Z. Feuerstein, "Carvedilol prevents severe hypertensive cardiomyopathy and remodeling," *Journal of Hypertension*, vol. 16, no. 6, pp. 871–884, 1998.

[23] Q. Xu, A. Chakravorty, R. A. D. Bathgate, A. M. Dart, and X.-J. Du, "Relaxin therapy reverses large artery remodeling and improves arterial compliance in senescent spontaneously hypertensive rats," *Hypertension*, vol. 55, no. 5, pp. 1260–1266, 2010.

[24] M. Cebova and F. Kristek, "Age-dependent ultrastructural changes of coronary artery in spontaneously hypertensive rats," *General Physiology and Biophysics*, vol. 30, no. 4, pp. 364–372, 2011.

[25] W. W. Brooks, C. H. Conrad, K. G. Robinson, W. S. Colucci, and O. H. L. Bing, "L-arginine fails to prevent ventricular remodeling and heart failure in the spontaneously hypertensive rat," *American Journal of Hypertension*, vol. 22, no. 2, pp. 228–234, 2009.

[26] M. Tanaka, H. Fujiwara, T. Onodera, D. J. Wu, Y. Hamashima, and C. Kawai, "Quantitative analysis of myocardial fibrosis in normals, hypertensive hearts, and hypertrophic cardiomyopathy," *British Heart Journal*, vol. 55, no. 6, pp. 575–581, 1986.

[27] A. M. Heagerty, C. Aalkjaer, S. J. Bund, N. Korsgaard, and M. J. Mulvany, "Small artery structure in hypertension: dual processes of remodeling and growth," *Hypertension*, vol. 21, no. 4, pp. 391–397, 1993.

[28] R. Deegan and A. J. J. Wood, "β-receptor antagonism does not fully explain esmolol-induced hypotension," *Clinical Pharmacology and Therapeutics*, vol. 56, no. 2, pp. 223–228, 1994.

[29] S. L. Amaral and L. C. Michelini, "Effect of gender on training-induced vascular remodeling in SHR," *Brazilian Journal of Medical and Biological Research*, vol. 44, no. 9, pp. 814–826, 2011.

[30] L. L. Wang, Y. Han, Z. Guo, S. Q. Han, and T. Liu, "Esmolol activates endogenous neurokinin activity inhibiting infarction-induced arrhythmias in rats: novel mechanisms of anti-arrhythmia," *Regulatory Peptides*, vol. 186, pp. 116–122, 2013.

[31] R. J. Sung, L. Blanski, J. Kirshenbaum, P. MacCosbe, P. Turlapaty, and A. R. Laddu, "Clinical experience with esmolol, a short-acting beta-adrenergic blocker in cardiac arrhythmias and myocardial ischemia," *Journal of Clinical Pharmacology*, vol. 26, pp. A15–A26, 1986.

[32] Y. Wang, M. Zhang, Y. Liu, Y. Liu, and M. Chen, "The effect of nebivolol on asymmetric dimethylarginine system in spontaneously hypertension rats," *Vascular Pharmacology*, vol. 54, no. 1-2, pp. 36–43, 2011.

[33] P. Szajerski, J. Zielonka, A. Sikora et al., "Radical scavenging and NO-releasing properties of selected beta-adrenoreceptor antagonists," *Free Radical Research*, vol. 40, no. 7, pp. 741–752, 2006.

[34] H. Horimoto, A. E. Saltman, G. R. Gaudette, and I. B. Krukenkamp, "Nitric oxide-generating β-adrenergic blocker nipradilol preserves postischemic cardiac function," *Annals of Thoracic Surgery*, vol. 68, no. 3, pp. 844–849, 1999.

[35] Z. Veresh, B. Debreczeni, J. Hamar, P. M. Kaminski, M. S. Wolin, and A. Koller, "Asymmetric dimethylarginine reduces nitric oxide donor-mediated dilation of arterioles by activating the vascular renin-angiotensin system and reactive oxygen species," *Journal of Vascular Research*, vol. 49, no. 4, pp. 363–372, 2012.

[36] S. Takai, K. Kirimura, D. Jin et al., "Significance of angiotensin II receptor blocker lipophilicities and their protective effect against vascular remodeling," *Hypertension Research*, vol. 28, no. 7, pp. 593–600, 2005.

[37] F. Feihl, L. Liaudet, B. I. Levy, and B. Waeber, "Hypertension and microvascular remodelling," *Cardiovascular Research*, vol. 78, no. 2, pp. 274–285, 2008.

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Clinical Study

Endothelial Function Increases after a 16-Week Diet and Exercise Intervention in Overweight and Obese Young Women

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Weight loss improves endothelial function in overweight individuals. The effects of weight loss through combined aerobic and resistance training and caloric restriction on in vivo vascular measures and blood markers associated with the regulation of endothelial function have not been comprehensively examined. Therefore, we investigated brachial artery endothelial function and potential regulatory blood markers in twenty overweight women (30.3 ± 2.0 years) who participated in 16 weeks of aerobic (5 d/wk) and resistance training (2 d/wk) (combined: ≥250 kcal/d) and caloric restriction (−500 kcal/d versus requirement). Resting brachial artery flow mediated dilation (FMD) and circulating endothelin-1 (ET-1) and interleukin-6 (IL-6) were assessed at baseline and following the intervention. Relative and absolute FMD increased (before: $4.0 \pm 0.5\%$ versus after: $6.9 \pm 0.6\%$, $P < 0.05$, and before: 0.14 ± 0.02 mm versus after: 0.23 ± 0.02 mm, $P < 0.05$, resp.), while body mass decreased (before: 86.9 ± 2.4 kg versus after: 81.1 ± 2.4 kg, $P < 0.05$) following the intervention. There were no changes in either blood marker (IL-6: before: 1.5 ± 0.2 pg/mL versus after: 1.5 ± 0.1 pg/mL, $P > 0.05$, and ET-1: before: 0.55 ± 0.05 pg/mL versus after: 0.59 ± 0.09 pg/mL, $P > 0.05$). 16 weeks of combined aerobic/resistance training and diet-induced weight loss improved endothelial function in overweight and obese young women, but this increase was not associated with changes in blood markers of vasoconstriction or inflammation.

1. Introduction

The vascular endothelium plays many roles, including contributing to the regulation of vascular smooth muscle tone. Endothelial dilatory capacity is commonly investigated non-invasively using the flow-mediated dilation (FMD) test. FMD measured in the brachial artery has been documented to correlate with pharmacological evaluations of coronary artery endothelial function [1]. Endothelial dysfunction may represent an early subclinical event in the development and progression of atherogenesis and is a known independent indicator of cardiovascular disease (CVD) [2, 3]. Aerobic exercise training improves endothelial function [4] and studies have reported a positive relationship between exercise-induced weight loss and endothelial function in overweight and obese men and women with established coronary heart disease [5]. A positive relationship between diet-induced weight loss and endothelial function in overweight and obese women with increased cardiovascular risk has also been reported [6–8]. No data exists on the effects of combined diet- and exercise-induced (aerobic and resistance) weight loss on FMD in otherwise healthy overweight and obese women.

At the molecular level, endothelial regulatory substances such as endothelin-1, a potent vasoconstrictor, and interleukin-6 (IL-6), a proinflammatory cytokine, have been measured in a number of exercise training studies [9–11]. Oxidative stress and inflammation are increased in overweight and obesity [12, 13]. As a result of increased oxidative stress, vascular inflammation also develops with obesity, as indicated by increases in expression of proinflammatory cytokines [12, 13]. Changes in endothelial function could be a key mechanistic link in the previously observed association between CVD and inflammation, as chronic inflammation has also been linked to endothelial dysfunction [9]. Specifically, proinflammatory cytokines may induce vasoconstriction by causing increased synthesis of endothelin-1 (ET-1) [9]. Although endothelial dysfunction is thought to be associated with a reduction in nitric oxide (NO), it has not been determined if increased ET-1 is a major contributor to this process. Increased ET-1 may contribute to the reduction of

NO bioavailability, which is often observed with endothelial dysfunction [10].

IL-6 has been linked to various pathological states, including obesity [14–16], and is secreted from a variety of different cells, including vascular endothelial cells [17]. IL-6 is a mediator of the acute inflammatory response and contributes to chronic inflammation in obesity [18, 19]. Elevations in IL-6 are thought to stimulate the synthesis of ET-1 in the vasculature [9]. Approximately 33% of total IL-6 originates from adipose tissue [12] and it may play a key role in the relationship between adiposity, inflammation, and CVD. Importantly, an inverse correlation between IL-6 concentrations and endothelial function has been observed [11].

Aerobic exercise training leads to improved arterial health in many populations [20] and is involved in cardiovascular risk reduction. Research examining the effects of resistance training on arterial structure and function has, however, yielded conflicting results [21, 22] and the effects of combined aerobic and resistance training on arterial structure and function have not been comprehensively examined [23, 24].

The purpose of this study was to investigate the effects of a 16-week combined aerobic and resistance training program and hypocaloric diet on endothelial function, assessed with brachial FMD and circulating ET-1 and IL-6 in otherwise healthy overweight and obese young women. We hypothesized that brachial FMD would increase and that this would be inversely related to changes in circulating ET-1 and IL-6, suggesting these circulating markers may act as alternative indices of vascular endothelial function.

2. Methods

2.1. Participants. This study was part of a larger lifestyle intervention study: Improving Diet, Exercise, and Lifestyle (I.D.E.A.L) for Women study, which involved 90 participants [25]. A small subset of participants ($n = 20$) from the larger lifestyle intervention study volunteered to participate in the cardiovascular measurements for the current study, based on participant availability and consent. The Research Ethics Board of Hamilton Health Sciences approved the study. Twenty young, overweight female subjects with an average age of 30.3 ± 2.0 years (mean ± SE) participated in this study. Participants were otherwise healthy, overweight, or obese women (BMI: 32.4 ± 0.8 kg/m^2). Other general inclusion criteria were sedentary lifestyle, regular menstrual cycle, and no vitamin or mineral supplementation. Participants were deemed healthy and thus eligible to participate based on their responses to a short medical screening questionnaire and measurement of serum lipids, glucose, and insulin concentrations all of which were normal (data not shown). All participants provided written informed consent before participating in the study.

2.2. Intervention

2.2.1. Diet. The targeted total daily energy reduction throughout the study was −750 kcal/d (500 kcal/d by diet and 250 kcal/d through exercise). All participants received individualized diet counseling by study dieticians and research nutritionists on a biweekly basis. Every 2 weeks, participants provided a 3-day food record to track compliance with the intervention [25].

2.2.2. Exercise Training. Participants completed 16 weeks of combined aerobic and resistance training as part of a targeted body composition-changing protocol. Participants exercised at the main fitness center at McMaster University. They engaged in various modes of aerobic exercise (stationary cycling, jogging on a treadmill, and walking on an indoor track) 5 d/wk and resistance exercise 2 d/wk with supervision. Each exercise session was designed to result in a minimum expenditure of 250 kcal in an effort to create a realistic workout routine for this population. During the week (Monday–Friday), subjects reported to the study office and were given a SenseWear Pro (BodyMedia, Pittsburgh, PA, US) arm band device to track energy expenditure [26]. Participants were requested to wear the SenseWear Pro device at home on several occasions randomly throughout the study in order to assess compliance with weekend workouts. The aerobic and resistance training programs have been described previously [25]. Briefly, participants engaged in a 2 d/wk resistance training protocol (upper body, lower body split). Weight progressions were made once the participants were able to successfully complete 3 sets of 10 repetitions at a given weight.

2.3. Cardiovascular Measurements. Testing sessions began with 10 minutes of supine rest to ensure representative resting measurements prior to the commencement of the vascular assessment. Continuous measurements of heart rate *via* single lead electrocardiograph (ECG) (model ML 123, ADInstruments Inc., Colorado Springs, Colorado, USA) and brachial blood pressure (BP) measurements *via* an automated applanation tonometer with oscillometric cuff calibration (model CBM-7000, Colin Medical Instruments, San Antonio, TX) were made. A FMD test was conducted to assess brachial artery endothelium-dependent function on the basis of previously established guidelines [27, 28]. All analogue signals (including those described below) were converted to digital by fast Fourier transform and sampled simultaneously at a sampling rate of 200 Hz using a commercially available data acquisition system (Power lab model ML 795, ADInstruments, Colorado Springs, Colorado, USA) and software program (LabChart 7.0, ADInstruments Inc., Colorado Springs, Colorado, USA).

2.4. Assessment of Flow-Mediated Dilation. FMD was assessed as previously described [29]. Briefly, with the participant in the supine position, the right arm was positioned and stabilized so that an optimal image of the brachial artery could be obtained in a comfortable position. An inflatable cuff was placed on the forearm, below the medial epicondyle [30], and remained deflated while baseline data were collected. Longitudinal B-mode ultrasound images of the left brachial artery were collected for five cardiac cycles using a 10-MHz linear array probe (System FiVe, GE Medical

Systems, Horten, Norway) positioned 3–5 cm proximal to the antecubital fossa at a frame rate of 11 frames/second. Following acquisition of the B-mode image 30 seconds of continuous blood velocity in the brachial artery were acquired using pulsed-wave mode Doppler at a frequency of 4 MHz with the sample volume width set to insonate the entire artery. The forward and reverse audio signals from the pulse wave mode Doppler spectrum were processed by an external spectral analysis system (Neurovision 500 M, Multigon Industries, Yonkers, NY) and an intensity-weighted calculated mean signal was acquired (Powerlab model ML 795). This system applies a fast Fourier transformation to the raw audio signals to determine continuous intensity weighted mean blood velocity (MBV). The MBV was sampled at 200 Hz during the FMD tests using commercially available hardware (Powerlab model ML 795, ADInstruments).

To create the flow stimulus, a forearm cuff was instantaneously inflated, using a rapid cuff inflator (model E20 and AG101, Hokanson, Bellevue, WA) to a standardized, supra-systolic pressure of 200 mmHg to ensure arterial inflow occlusion and ischemia of downstream vessels and tissue [27]. The cuff was instantaneously deflated after 5 min. of occlusion and during the first 90 s after cuff release reactive hyperemic intensity weighted mean blood velocity signals were obtained as described above, followed by a B-mode image of the left brachial artery for 12 cardiac cycles at a frame rate of 11 frames/second. B-mode images were stored in Digital Imaging and Communications in Medicine (DICOM) format for later offline editing and analysis.

3. Data Analysis

3.1. Brachial Artery Blood Velocity. Preocclusion and postocclusion MBV were analyzed offline using LabChart 7 Pro for Windows (Powerlab ML 795, ADInstruments) in 3 s average time bins after correcting for angle of insonation (all $\leq 68^{\circ}$). Mean blood flow (preocclusion) was determined by multiplying brachial artery cross-sectional area by MBV. Recently published FMD guidelines advocate the normalization of FMD responses to the entire reactive hyperemic stimulus rather than normalizing to the peak stimulus [28]. The following equation was used to calculate shear rate (SR) for each participant for each 3 s bin [31]:

$$\text{Shear Rate} = 8 \times \left(\frac{\text{Velocity}}{\text{Diameter}} \right), \tag{1}$$

where velocity represents the mean of the velocity profile in 3 s bins for the first 90 s postcuff release and the baseline brachial diameter (mm) is used for the artery diameter value. The area under the curve of the shear rate was calculated from the mean of the first point, using the trapezoid rule to obtain the area under the entire curve (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA). The entire reactive hyperemic stimulus was quantified as the shear rate area under the curve (AUC).

3.2. Brachial Artery Diameter. Using commercially available software (SanteDICOM Editor, Version 3.0.12, Santesoft, Athens, Greece), the end-diastolic frames, determined by the R-spike of the ECG trace, were extracted and stacked in a new DICOM file for determination of brachial artery diameters. A semiautomated edge detection software program (Artery Measurement System, Image and Data Analysis, Tomas Gustavsson, gustav@alumni.chalmers.se) was used to detect the vessel diameters within a specific region of interest. The program identifies the borders of the arterial wall within a selected region of interest on the basis of the contrasting intensity of brightness between the arterial wall and lumen, and it determines the diameter from approximately 100 points of measurement within the region of interest. Preocclusion diameters were determined from the average of the five end-diastolic frames. Peak postocclusion FMD diameter was determined from the 12 end-diastolic frames. From this data, the absolute FMD (mm) and relative FMD (%FMD) were calculated as follows [27]:

$$\begin{aligned} \text{Absolute FMD} &= \text{Peak Diameter (mm)} \\ &\quad - \text{Baseline Diameter (mm)} \\ \text{Relative FMD} &= \left(\frac{\text{Absolute FMD}}{\text{Baseline Diameter}} \right) \times 100\%. \end{aligned} \tag{2}$$

Relative FMD (%FMD) was normalized to the area under the entire SR curve and reported as

$$\text{Normalized FMD} = \left(\frac{\%\text{FMD}}{\text{SR}_{\text{AUC}}} \right). \tag{3}$$

3.3. Blood Analysis. Overnight fasted venous blood samples were collected prior to and after 16 weeks of the intervention for further analysis. Serum was stored at -20°C for further analysis. Serum samples were analyzed using ELISAs for concentrations of interleukin-6 (R&D Systems Quantikine, Minneapolis, MN) and endothelin-1 (Enzo, Life Sciences Assay Designs, Farmingdale, NY) using the corresponding immunoassay kits.

3.4. Statistics. Results are presented as mean ± SE and differences were considered significant at $P < 0.05$. Data was analyzed using SPSS (Version 20). Mean differences were analyzed using Student's t-tests. Pearson correlations were used to assess relationships between flow-mediated dilation measures and blood markers.

4. Results

4.1. Participants. A total of twenty ($n = 20$) young healthy women (age = 30.3 ± 2.0 years; height = 163 ± 1 cm) completed the laboratory vascular testing. Body mass and BMI decreased after the intervention (Table 1).

4.2. Vascular Measures. There was no change in resting heart rate or resting mean arterial pressure (MAP), systolic blood pressure (SBP), or diastolic blood pressure (DBP) over time following the intervention (Table 2).

Table 1: Body composition measures before and following the 16-week intervention.

	Before (mean ± SE)	After (mean ± SE)	P value
Weight (kg)	86.8 ± 2.4	80.6 ± 2.4	<0.001
BMI (kg/m^2)	32.4 ± 0.8	30.1 ± 0.7	<0.001

Table 2: Resting vascular measures before and following the 16-week intervention.

Resting variable	Before (mean ± SE)	After (mean ± SE)
Brachial diameter (mm)	3.39 ± 0.08	3.48 ± 0.09
HR (bpm)	65 ± 1	62 ± 1
MAP (mmHg)	81 ± 2	81 ± 3
SBP (mmHg)	116 ± 2	114 ± 3
DBP (mmHg)	63 ± 2	63 ± 13

4.3. Arterial Function. Relative (Figure 1(a)) and absolute FMD (Figure 1(b)) increased after the 16-week diet and exercise intervention. Data was pooled to represent the entire population before and after the intervention (relative: before: 4.0 ± 0.5% versus after: 6.9 ± 0.6%, $P = 0.005$; absolute: before: 0.14 ± 0.02 mm versus after: 0.23 ± 0.02 mm, $P = 0.004$). However, when FMD was normalized to shear rate, it was unchanged after 16 weeks (normalized: before: $1.8 \times 10^4 \pm 4.8 \times 10^5$ versus after: $2.2 \times 10^4 \pm 2.3 \times 10^5$, $P = 0.45$; Figure 1(c)). There were no relationships observed between weight loss (difference in body mass before and after) and increased FMD (relative, absolute, or normalized) following the intervention (weight loss versus change in absolute FMD: $r = -0.118$, $P = 0.620$; weight loss versus change in relative FMD: $r = -0.070$, $P = 0.768$; weight loss versus change in normalized FMD: $r = -0.172$, $P = 0.468$).

4.4. Arterial Structure. There was no change in resting brachial artery diameter after the 16-week diet and exercise intervention (Table 2).

4.5. Blood Markers. There was no change in either of the serum markers (IL-6: before = 1.5 ± 0.2 pg/mL and after = 1.5 ± 0.1 pg/mL, $P = 0.58$, and ET-1: before = 0.55 ± 0.05 pg/mL and after = 0.59 ± 0.09 pg/mL, $P = 0.73$; $N = 19$, as one participant had undetectable ET-1 values). There were no relationships observed between any measures of FMD and IL-6 (Figure 2) or ET-1 ($N = 19$) before or after the intervention (Figure 3). One participant was removed from this analysis, as her ET-1 concentrations were undetectable.

5. Discussion

The main findings of this study were that brachial artery endothelial function assessed by relative (Δ%) and absolute (Δmm) FMD improved after the 16-week diet and exercise intervention; however, circulating serum concentrations of IL-6 and ET-1 were unchanged in our population of overweight women. Given the importance of endothelial function as an independent risk factor for CVD [2, 3] we view our findings as a relevant demonstration of what a multifaceted exercise- and diet-based weight loss intervention can do to alleviate CVD risk. The women included in this study were by definition sedentary [32, 33] which may have contributed to a reduction in baseline endothelial function. The exercise component of our intervention was therefore likely responsible, at least in part, for the improved endothelial function we observed.

Interestingly, while all of our subjects lost weight, the weight loss was not associated with changes in FMD or with reductions in markers of inflammation and so it is difficult to directly ascribe the changes in FMD to changes in body weight or indirectly through the influence this might have had on inflammation. Weight loss, with either diet [7, 8] and/or aerobic exercise [5] interventions, has previously been linked with increased endothelial function as measured by brachial FMD in overweight and obese women. While we did observe an improvement in FMD over time, there was no significant relationship between weight loss and improved endothelial function via brachial FMD measures with our combined diet and exercise intervention. Changes in FMD (%) with diet-induced weight-loss have been previously demonstrated; however, the reported differences are larger in magnitude than those observed in the current study. Mavri et al. (2011) observed an increase from 7.7% to 12.4% after 5 months of a low calorie diet in middle-aged obese women, whereas we observed an increase in FMD of 4.0% to 6.9% after 4 months. We do not singularly attribute the changes in FMD to the weight loss of the individuals or the dietary component of the intervention but rather to the combined vascular stimulus provided by the exercise and dietary intervention.

Aerobic exercise training has been shown to improve endothelial function in a variety of populations [34] including overweight postmenopausal women [22, 35]. We found a significant increase in FMD in overweight premenopausal women who performed combined aerobic and resistance training for 16 weeks. Our findings agree with those of Kwon and colleagues [22] who reported increases in relative FMD after a 12-week aerobic training program in overweight postmenopausal women ($P = 0.032$). Other studies have also shown increases in endothelial function as assessed by FMD using combined aerobic and resistance training interventions in other populations including young healthy men and men and women with type 2 diabetes mellitus [23, 24]. Our % change values are comparable to those found in the existing literature after combined aerobic and resistance training, suggesting the vascular stimulus from the exercise program may be the dominant mechanism associated with our observed increase in FMD. To our knowledge there are no studies to date that have investigated the effects of a combined diet and aerobic and resistance program on FMD in young overweight women and further research is needed to delineate the mechanisms associated with these changes.

Mechanistically, ET-1 and IL-6 are potential regulators of endothelial function. Inflammation has the ability to impair FMD as cytokines which may lead to increased vasoconstriction [9]. IL-6 contributes to chronic inflammation in conditions such as obesity and studies have identified

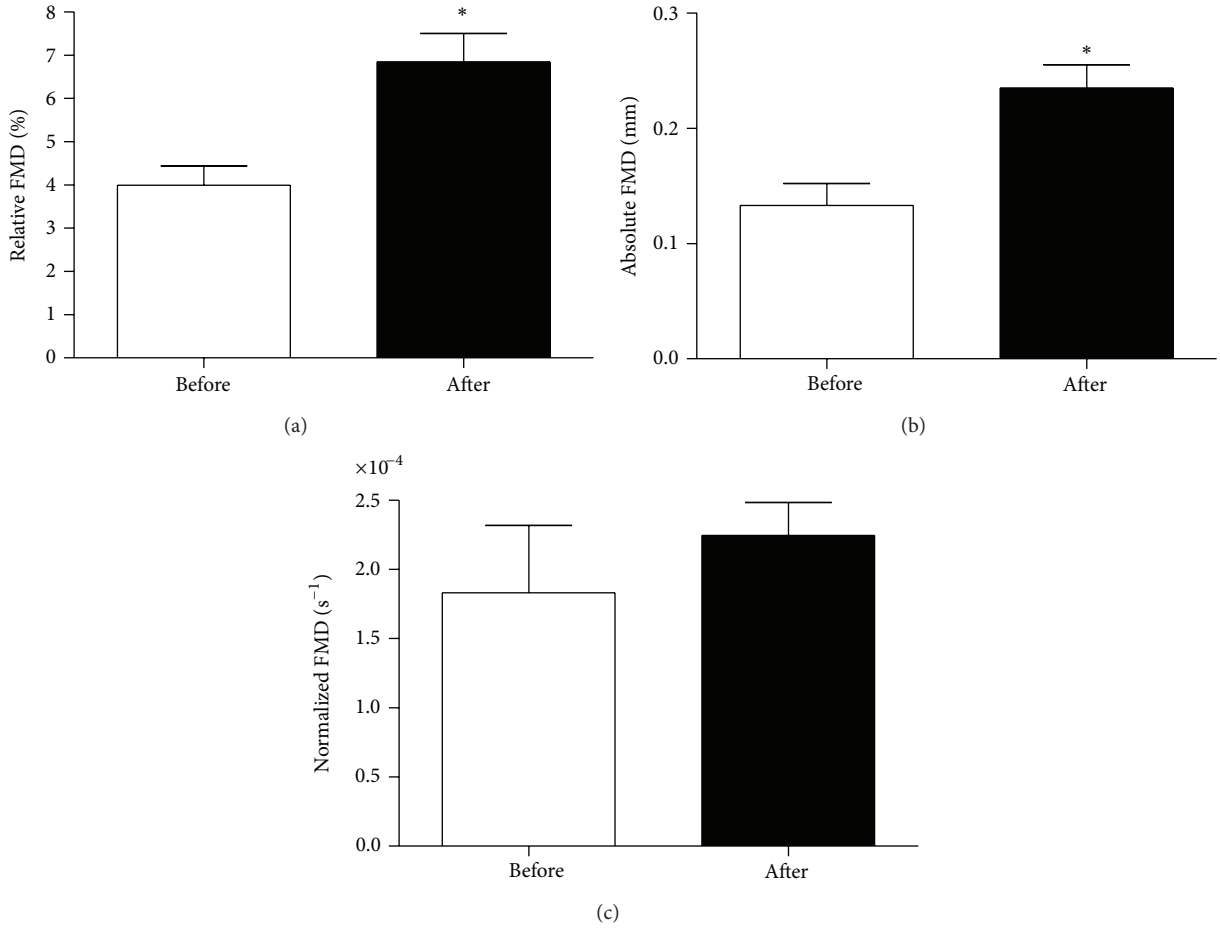

Figure 1: Changes in FMD from Week 0 to Week 16.

elevated IL-6 levels in obese individuals [18, 19]. Chronic inflammation has been linked to endothelial dysfunction [9], and thus a decrease in endothelial dysfunction may be related to changes in inflammatory markers. Inverse relationships ($r = -0.123$, $P < 0.0001$) have been reported between serum IL-6 concentration and endothelial function [11]. ET-1 can decrease NO bioavailability either by decreasing its production or by increasing its degradation [10]. However, in the current study, ET-1 and IL-6 were unchanged following the 16-week intervention and there was no relationship observed between IL-6 or ET-1 and any of the FMD measurements suggesting that in our subject population IL-6 and ET-1 are not sensitive markers for changes in endothelial function observed during this type of lifestyle intervention.

Another potential explanation for our observation of no change in IL-6 in conjunction with the observed increase in FMD is the inclusion of resistance training in the exercise regime for our participants. There are conflicting results in the literature with respect to the effects of resistance training on circulating markers of inflammation. A recent study by Patterson et al. demonstrated that IL-6 levels were elevated following resistance training in older men [36], while Phillips et al. observed no change in IL-6 after resistance training in postmenopausal women [37]. It is also possible that the women in this study did not have chronic inflammation and thus we are observing a "floor" effect where even an intervention involving weight loss and exercise would not result in decreases in markers of inflammation.

It appears that ET-1 generally decreases following aerobic exercise training [38–40]. A recent study showed that ET-1 is reduced after just three weeks of aerobic training in middle-aged obese type 2 diabetic men and women [38]. Kasmay and colleagues (2010) observed a decrease in ET-1 when aerobic exercise training was accompanied with a low-calorie diet in patients with impaired glucose tolerance [39]. Maeda and colleagues (2003) observed a reduction in plasma ET-1 concentrations in older healthy women after 3 months of aerobic training [40]. In contrast we observed no differences in ET-1 concentration following 16 weeks of our exercise and diet intervention. While the populations involved in these studies were different than the current study, all were overweight and some were middle-aged women, thereby facilitating comparisons. It is possible the resistance-training component in our intervention attenuated any aerobic exercise training stimulated decreases in ET-1. Very little information exists regarding the effects of resistance training on ET-1; however, Maeda et al. (2004) observed a decrease in plasma ET-1 in young healthy men after 8 weeks of resistance training [41].

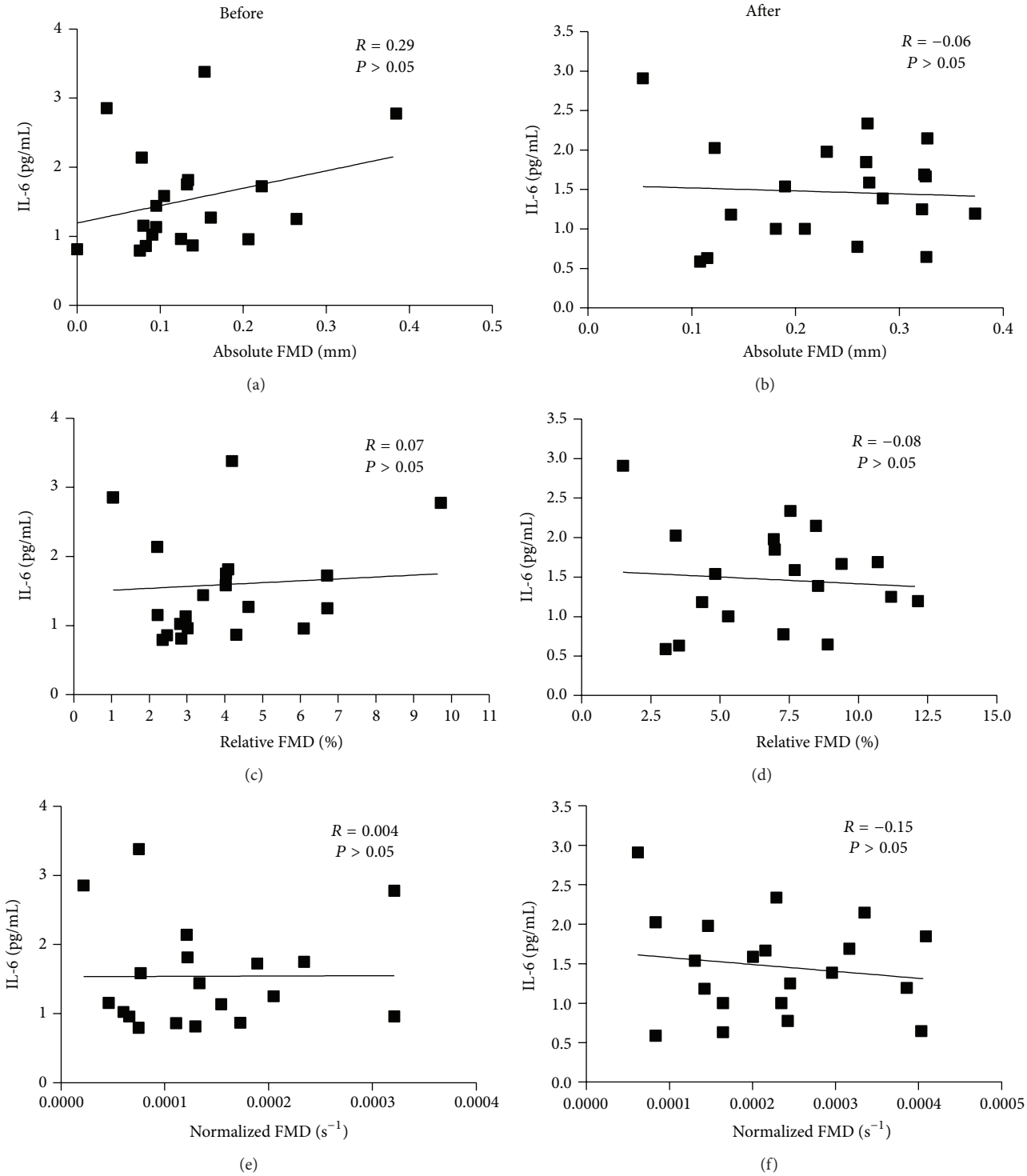

Figure 2: Relationships between FMD and IL-6 at Week 0 and Week 16.

In this study we found no change in ET-1 after resistance training. More studies are necessary to better understand the relationship between ET-1 and resistance exercise training.

It is important to address the time course of changes in vascular structure and function with exercise interventions. It has been suggested that short-term (2–6 weeks) exercise training enhances eNOS and NO activity [42–46] while long-term (>8 weeks) training induces structural changes [46–48], marked by an increase in resting arterial diameter. This arterial remodeling is thought to normalize shear rate levels and thus may result in NO-mediated endothelial function returning to pretraining levels [34, 49, 50]. Original animal

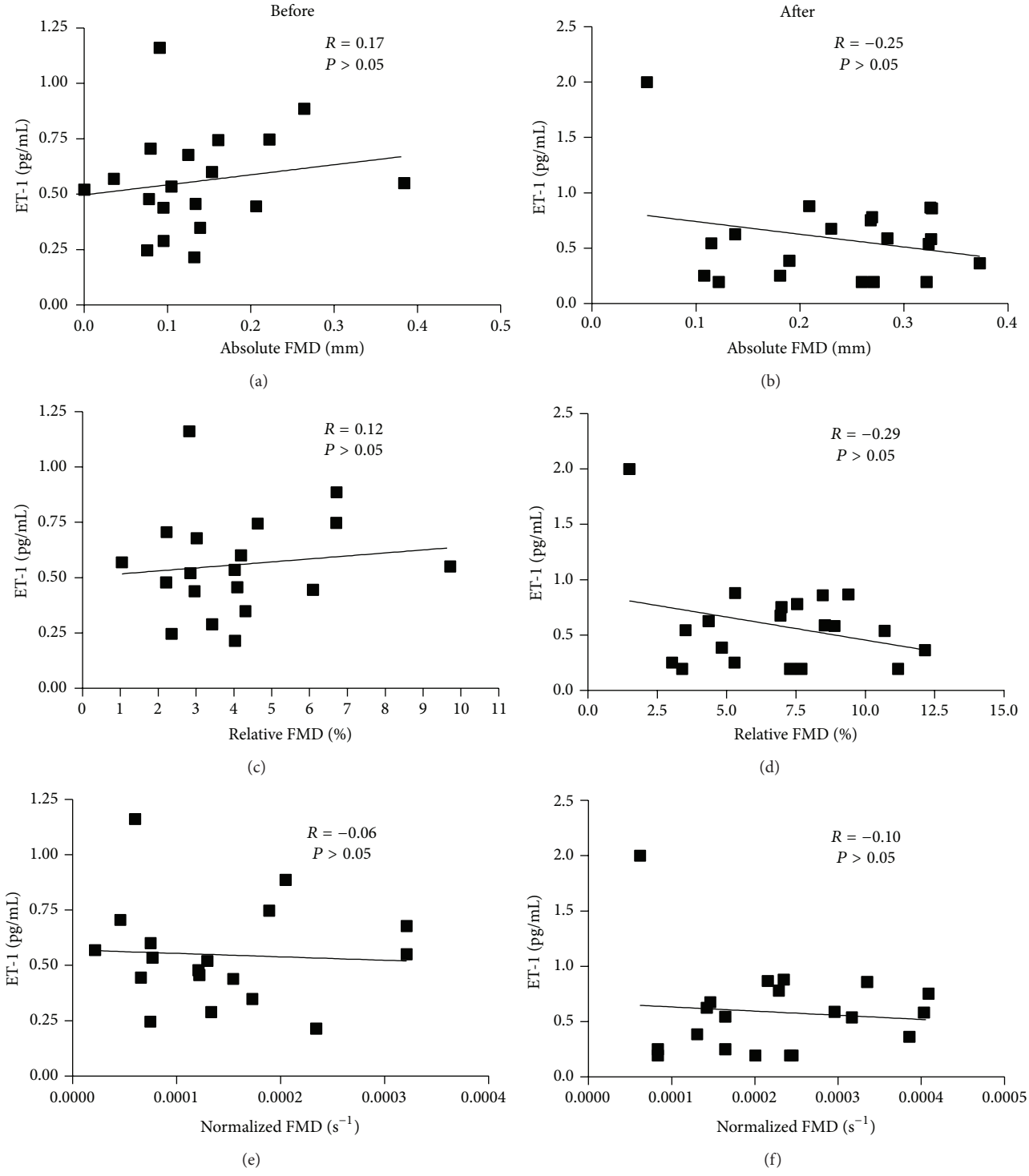

Figure 3: Relationships between FMD and ET-1 at Week 0 and Week 16.

studies by Laughlin et al. [49, 50] and a compilation of data from different populations [46, 51–56] support this proposed time course of vascular function (peak at 6 weeks) and structure (>8 weeks) changes. In our study, FMD was still elevated at the 16-week time point, while resting brachial diameter was unchanged indicating that the time course of vascular changes with exercise may be different in overweight and obese young women and may also be influenced by the details of the intervention, including exercise mode, duration, intensity, and dietary modifications.

In conclusion, we observed an increase in endothelial function as measured by absolute and relative FMD following 16 weeks of a diet and exercise intervention that resulted in weight loss in overweight and obese premenopausal women.

No changes were observed in blood markers, IL-6 and ET-1, which are often proposed as being mechanistically relevant in inflammation and vasoconstriction. Our study demonstrates that endothelial function is improved with weight loss and combined aerobic and resistance exercise and may be helpful for designing lifestyle interventions for overweight individuals at elevated CVD risk. Further research is warranted to better understand the mechanisms responsible for the observed changes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lisa M. Cotie prepared the paper and collected and analyzed all vascular and blood data; Andrea R. Josse contributed to study design and editing of the paper and was the lead student investigator on the larger IDEAL study; Stuart M. Phillips contributed to study design and editing of the paper and was the lead investigator on the larger IDEAL study; Maureen J. MacDonald contributed to study design and editing of the paper.

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References

[1] H. Teragawa, K. Ueda, K. Matsuda et al., "Relationship between endothelial function in the coronary and brachial arteries," *Clinical Cardiology*, vol. 28, no. 10, pp. 460–466, 2005.

[2] P. O. Bonetti, L. O. Lerman, and A. Lerman, "Endothelial dysfunction: a marker of atherosclerotic risk," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 2, pp. 168–175, 2003.

[3] J. A. Vita and J. F. Keaney Jr., "Endothelial function: a barometer for cardiovascular risk?" *Circulation*, vol. 106, no. 6, pp. 640–642, 2002.

[4] M. A. Black, N. T. Cable, D. H. J. Thijssen, and D. J. Green, "Impact of age, sex, and exercise on brachial artery flow-mediated dilatation," *American Journal of Physiology*, vol. 297, no. 3, pp. H1109–H1116, 2009.

[5] P. A. Ades, P. D. Savage, S. Lischke et al., "The effect of weight loss and exercise training on flow-mediated dilatation in coronary heart disease: a randomized trial," *Chest*, vol. 140, no. 6, pp. 1420–1427, 2011.

[6] S. Buscemi, L. Cosentino, G. Rosafio et al., "Effects of hypocaloric diets with different glycemic indexes on endothelial function and glycemic variability in overweight and in obese adult patients at increased cardiovascular risk," *Clinical Nutrition*, vol. 32, no. 3, pp. 346–352, 2013.

[7] S. J. Bigornia, M. M. Mott, D. T. Hess et al., "Long-term successful weight loss improves vascular endothelial function in severely obese individuals," *Obesity*, vol. 18, no. 4, pp. 754–759, 2010.

[8] A. Mavri, P. Poredoš, D. Šuran, B. Gaborit, I. Juhan-Vague, and P. Poredoš, "Effect of diet-induced weight loss on endothelial dysfunction: early improvement after the first week of dieting," *Heart and Vessels*, vol. 26, no. 1, pp. 31–38, 2011.

[9] E. Vila and M. Salaices, "Cytokines and vascular reactivity in resistance arteries," *American Journal of Physiology*, vol. 288, no. 3, pp. H1016–H1021, 2005.

[10] M. Iglarz and M. Clozel, "Mechanisms of ET-1-induced endothelial dysfunction," *Journal of Cardiovascular Pharmacology*, vol. 50, no. 6, pp. 621–628, 2007.

[11] J. A. Vita, J. F. Keaney Jr., M. G. Larson et al., "Brachial artery vasodilator function and systemic inflammation in the framingham offspring study," *Circulation*, vol. 110, no. 23, pp. 3604–3609, 2004.

[12] V. Mohamed-Ali, S. Goodrick, A. Rawesh et al., "Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-α, in vivo," *Journal of Clinical Endocrinology and Metabolism*, vol. 82, no. 12, pp. 4196–4200, 1997.

[13] J. S. Yudkin, M. Kumari, S. E. Humphries, and V. Mohamed-Ali, "Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link?" *Atherosclerosis*, vol. 148, no. 2, pp. 209–214, 2000.

[14] C. P. Lambert, N. R. Wright, B. N. Finck, and D. T. Villareal, "Exercise but not diet-induced weight loss decreases skeletal muscle inflammatory gene expression in frail obese elderly persons," *Journal of Applied Physiology*, vol. 105, no. 2, pp. 473–478, 2008.

[15] V. M. Mendoza-Núñez, Á. García-Sánchez, M. Sánchez-Rodríguez, R. E. Galván-Duarte, and M. E. Fonseca-Yerena, "Overweight, waist circumference, age, gender, and insulin resistance as risk factors for hyperleptinemia," *Obesity Research*, vol. 10, no. 4, pp. 253–259, 2002.

[16] A. E. Schutte, H. W. Huisman, R. Schutte et al., "Adipokines and cardiometabolic function: how are they interlinked?" *Regulatory Peptides*, vol. 164, no. 2-3, pp. 133–138, 2010.

[17] M. Rattazzi, M. Puato, E. Faggin, B. Bertipaglia, A. Zambon, and P. Pauletto, "C-reactive protein and interleukin-6 in vascular disease: culprits or passive bystanders?" *Journal of Hypertension*, vol. 21, no. 10, pp. 1787–1803, 2003.

[18] L. U. Monzillo, O. Hamdy, E. S. Horton et al., "Effect of lifestyle modification on adipokine levels in obese subjects with insulin resistance," *Obesity Research*, vol. 11, no. 9, pp. 1048–1054, 2003.

[19] S. K. Fried, D. A. Bunkin, and A. S. Greenberg, "Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid," *Journal of Clinical Endocrinology and Metabolism*, vol. 83, no. 3, pp. 847–850, 1998.

[20] A. Miyaki, S. Maeda, M. Yoshizawa et al., "Effect of habitual aerobic exercise on body weight and arterial function in overweight and obese men," *American Journal of Cardiology*, vol. 104, no. 6, pp. 823–828, 2009.

[21] M. Rakobowchuk, C. L. McGowan, P. C. De Groot, J. W. Hartman, S. M. Phillips, and M. J. MacDonald, "Endothelial function

of young healthy males following whole body resistance training," *Journal of Applied Physiology*, vol. 98, no. 6, pp. 2185–2190, 2005.

[22] H. R. Kwon, K. W. Min, H. J. Ahn et al., "Effects of aerobic exercise vs. resistance training on endothelial function in women with type 2 diabetes mellitus," *Diabetes & Metabolism Journal*, vol. 35, no. 4, pp. 364–373, 2011.

[23] T. Okamoto, M. Masuhara, and K. Ikuta, "Combined aerobic and resistance training and vascular function: effect of aerobic exercise before and after resistance training," *Journal of Applied Physiology*, vol. 103, no. 5, pp. 1655–1661, 2007.

[24] A. Maiorana, G. O'Driscoll, C. Cheetham et al., "The effect of combined aerobic and resistance exercise training on vascular function in type 2 diabetes," *Journal of the American College of Cardiology*, vol. 38, no. 3, pp. 860–866, 2001.

[25] A. R. Josse, S. A. Atkinson, M. A. Tarnopolsky, and S. M. Phillips, "Increased consumption of dairy foods and protein during diet- and exercise-induced weight loss promotes fat mass loss and lean mass gain in overweight and obese premenopausal women," *Journal of Nutrition*, vol. 141, no. 9, pp. 1626–1634, 2011.

[26] G. J. Welk, J. J. McClain, J. C. Eisenmann, and E. E. Wickel, "Field validation of the MTI actigraph and bodymedia armband monitor using the IDEEA monitor," *Obesity*, vol. 15, no. 4, pp. 918–928, 2007.

[27] M. C. Corretti, T. J. Anderson, E. J. Benjamin et al., "Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the international brachial artery reactivity task force," *Journal of the American College of Cardiology*, vol. 39, no. 2, pp. 257–265, 2002.

[28] D. H. Thijssen, M. A. Black, K. E. Pyke et al., "Assessment of flow-mediated dilation in humans: a methodological and physiological guideline," *American Journal of Physiology*, vol. 300, no. 1, pp. H2–H12, 2011.

[29] A. A. Martin, L. M. Cotie, B. W. Timmons et al., "Arterial structure and function in ambulatory adolescents with cerebral palsy are not different from healthy controls," *International Journal of Pediatrics*, vol. 2012, Article ID 168209, 8 pages, 2012.

[30] A. E. Donald, M. Charakida, T. J. Cole et al., "Non-invasive assessment of endothelial function. Which technique?" *Journal of the American College of Cardiology*, vol. 48, no. 9, pp. 1846–1850, 2006.

[31] B. A. Parker, T. L. Trehearn, and J. R. Meendering, "Pick your Poiseuille: normalizing the shear stimulus in studies of flow-mediated dilation," *Journal of Applied Physiology*, vol. 107, no. 4, pp. 1357–1359, 2009.

[32] R. R. Pate, J. R. O'Neill, and F. Lobelo, "The evolving definition of 'edentary'," *Exercise and Sport Sciences Reviews*, vol. 36, no. 4, pp. 173–178, 2008.

[33] K. I. Proper, A. S. Singh, W. Van Mechelen, and M. J. M. Chinapaw, "Sedentary behaviors and health outcomes among adults: a systematic review of prospective studies," *American Journal of Preventive Medicine*, vol. 40, no. 2, pp. 174–182, 2011.

[34] D. J. Green, A. Maiorana, G. O'Driscoll, and R. Taylor, "Effect of exercise training on endothelium-derived nitric oxide function in humans," *Journal of Physiology*, vol. 561, part 1, pp. 1–25, 2004.

[35] D. L. Swift, C. P. Earnest, S. N. Blair, and T. S. Church, "The effect of different doses of aerobic exercise training on endothelial function in postmenopausal women with elevated blood pressure: results from the DREW study," *British Journal of Sports Medicine*, vol. 46, no. 10, pp. 753–758, 2012.

[36] S. D. Patterson, M. Leggate, M. A. Nimmo, and R. A. Ferguson, "Circulating hormone and cytokine response to low-load resistance training with blood flow restriction in older men," *European Journal of Applied Physiology*, vol. 113, no. 3, pp. 713–719, 2013.

[37] M. D. Phillips et al., "Resistance training reduces subclinical inflammation in obese, postmenopausal women," *Medicine and Science in Sports and Exercise*, vol. 44, no. 11, pp. 2099–2110, 2012.

[38] P. Lucotti, L. D. Monti, E. Setola et al., "Aerobic and resistance training effects compared to aerobic training alone in obese type 2 diabetic patients on diet treatment," *Diabetes Research and Clinical Practice*, vol. 94, no. 3, pp. 395–403, 2011.

[39] Ö. Kasmay, N. Ergen, S. Bilsel et al., "Diet-supported aerobic exercise reduces blood endothelin-1 and nitric oxide levels in individuals with impaired glucose tolerance," *Journal of Clinical Lipidology*, vol. 4, no. 5, pp. 427–434, 2010.

[40] S. Maeda, T. Tanabe, T. Miyauchi et al., "Aerobic exercise training reduces plasma endothelin-1 concentration in older women," *Journal of Applied Physiology*, vol. 95, no. 1, pp. 336–341, 2003.

[41] S. Maeda, T. Miyauchi, M. Iemitsu, J. Sugawara, Y. Nagata, and K. Goto, "Resistance exercise training reduces plasma endothelin-1 concentration in healthy young humans," *Journal of Cardiovascular Pharmacology*, vol. 44, supplement 1, pp. S443–S446, 2004.

[42] M. D. Delp and M. H. Laughlin, "Time course of enhanced endothelium-mediated dilation in aorta of trained rats," *Medicine and Science in Sports and Exercise*, vol. 29, no. 11, pp. 1454–1461, 1997.

[43] M. D. Delp, R. M. McAllister, and M. H. Laughlin, "Exercise training alters endothelium-dependent vasoreactivity of rat abdominal aorta," *Journal of Applied Physiology*, vol. 75, no. 3, pp. 1354–1363, 1993.

[44] W. C. Sessa, K. Pritchard, N. Seyedi, J. Wang, and T. H. Hintze, "Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression," *Circulation Research*, vol. 74, no. 2, pp. 349–353, 1994.

[45] D. Sun, A. Huang, A. Koller, and G. Kaley, "Short-term daily exercise activity enhances endothelial NO synthesis in skeletal muscle arterioles of rats," *Journal of Applied Physiology*, vol. 76, no. 5, pp. 2241–2247, 1994.

[46] T. M. Tinken, D. H. J. Thijssen, M. A. Black, N. T. Cable, and D. J. Green, "Time course of change in vasodilator function and capacity in response to exercise training in humans," *Journal of Physiology*, vol. 586, no. 20, pp. 5003–5012, 2008.

[47] B. M. Prior, P. G. Lloyd, H. T. Yang, and R. L. Terjung, "Exercise-induced vascular remodeling," *Exercise and Sport Sciences Reviews*, vol. 31, no. 1, pp. 26–33, 2003.

[48] M. D. Brown, "Exercise and coronary vascular remodelling in the healthy heart," *Experimental Physiology*, vol. 88, no. 5, pp. 645–658, 2003.

[49] M. H. Laughlin, L. J. Rubin, J. W. E. Rush, E. M. Price, W. G. Schrage, and C. R. Woodman, "Short-term training enhances endothelium-dependent dilation of coronary arteries, not arterioles," *Journal of Applied Physiology*, vol. 94, no. 1, pp. 234–244, 2003.

[50] M. H. Laughlin, "Endothelium-mediated control of coronary vascular tone after chronic exercise training," *Medicine and Science in Sports and Exercise*, vol. 27, no. 8, pp. 1135–1144, 1995.

[51] B. A. Kingwell, K. L. Berry, J. D. Cameron, C. L. Jennings, and A. M. Dart, "Arterial compliance increases after moderate-intensity cycling," *American Journal of Physiology*, vol. 273, no. 5, pp. H2186–H2191, 1997.

[52] R. Bergholm, S. Mäkimattila, M. Valkonen et al., "Intense physical training decreases circulating antioxidants and endothelium-dependent vasodilatation in vivo," *Atherosclerosis*, vol. 145, no. 2, pp. 341–349, 1999.

[53] A. Maiorana, G. O'Driscoll, L. Dembo, C. Goodman, R. Taylor, and D. Green, "Exercise training, vascular function, and functional capacity in middle-aged subjects," *Medicine and Science in Sports and Exercise*, vol. 33, no. 12, pp. 2022–2028, 2001.

[54] A. Maiorana, G. O'Driscoll, L. Dembo et al., "Effect of aerobic and resistance exercise training on vascular function in heart failure," *American Journal of Physiology*, vol. 279, no. 4, pp. H1999–H2005, 2000.

[55] R. Hambrecht, A. Wolf, S. Gielen et al., "Effect of exercise on coronary endothelial function in patients with coronary artery disease," *The New England Journal of Medicine*, vol. 342, no. 7, pp. 454–460, 2000.

[56] A. Sciacqua, M. Candigliota, R. Ceravolo et al., "Weight loss in combination with physical activity improves endothelial dysfunction in human obesity," *Diabetes Care*, vol. 26, no. 6, pp. 1673–1678, 2003.

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Research Article

Age-Related Alterations in Endothelial Function of Femoral Artery in Young SHR and WKY Rats

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The present study was designed to evaluate the effects of vascular aging in juvenescence on endothelial function in femoral arteries and to assess differences between normotensive and hypertensive rats. The aim of the study was to determine if age affected nitric oxide- (NO-) mediated relaxations in normotensive and hypertensive rats. Juvenile (7-week-old) and young adult (22-week-old) male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) were used in this study. Femoral artery (FA) reactivity was determined by wire myograph and NO synthase activity by conversion of [^{3}H]-L-arginine. During juvenescence systolic blood pressure (tail-cuff) increased significantly only in SHR, while NO synthesis decreased significantly in both strains. Endothelium-dependent relaxations to acetylcholine were reduced in the FA of SHR compared to age-matched WKY at both ages, yet these parameters were unchanged in adult rats compared with juvenile animals. The NO-dependent component of vasorelaxation was markedly reduced, whereas the NO-independent component was increased in adult compared to juvenile rats in both strains. The endothelial dysfunction in SHR at both ages was associated with reduction of NO-independent mechanisms. In conclusion, aging in early periods of life was associated with reduction of vascular NO production and bioavailability in both strains investigated. This reduction was however fully compensated by accentuation of NO-independent mechanisms.

1. Introduction

Studies using experimental animal models as well as clinical research have indicated that hypertension is associated with endothelial dysfunction, a state in which the endothelial disorder leads to reduced vasodilation and increased vasoconstriction. Despite enormous research effort, the causal relationship between endothelial dysfunction and hypertension has remained unclear.

Endothelial dysfunction develops due to various risk factors, including aging of the organism. The latter, however, may be the causative agent even in the absence of established risk factors [1, 2]. Moreover, aging and hypertension have been identified as major risk factors for cardiovascular diseases. Advanced aging is associated with reduced endothelium-dependent relaxations, that is, endothelial dysfunction, in both human and animal arteries, promoting thus the initiation and development of cardiovascular diseases [3–8].

Vasorelaxation is primarily controlled by the endothelium, and both by production of endothelium-derived relaxing factors (EDRFs), as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2), and also by endothelium-derived contracting factors (EDCFs), which reduce vasorelaxation [9]. Disbalance between EDRFs and EDCFs results in an endothelial dysfunction, which has been observed in various diseased states, including hypertension [10]. Increased EDCFs-induced vasoconstriction, mediated presumably by cyclooxygenase (COX) metabolites, participates in impairment of endothelium-dependent vasorelaxation in both genetic models of arterial hypertension and age-induced vascular changes [5, 6, 10].

Regarding vasorelaxation, NO mediates many physiological and pathophysiological functions. Age-related vascular

dysfunction was shown to result mainly from reduced NO production [11, 12]. In human umbilical vein endothelial cells, aging decreased the production of NO and the activity and expression of eNOS protein [11]. Several studies indicated that aging blunted NO-dependent relaxations [8, 13–16]. All the reported studies, however, investigated vascular function of middle-aged and old (55-week- to 35-month-old) rats compared to young adult (12- to 24-week-old) rats. According to animal models and the vascular bed studied, aging-related changes can involve different mechanisms. Yet little information is available on early alterations in the NO-mediated vasorelaxation from blood vessels of juvenile and young adult rats either with or without a genetic load of hypertension.

As alterations in aging may, at least in part, depend on hemodynamic factors such as arterial pressure [14], a further aim of this study was to determine the influence of aging in young spontaneously hypertensive rats (SHR). We used SHR as this experimental model of genetic hypertension is similar to the human form of increased blood pressure (BP) and can help to better understand the mechanisms of essential hypertension in humans [17, 18]. The characteristic acceleration of BP rise in SHR (as compared with normotensive WKY rats) occurs mainly between the 3rd and 10th week of age. Over this period of age, their BP rapidly increases and continues to rise at least until the age of 20 weeks [19]. In contrast, the BP of WKY reaches adult levels by the 10th week of age [19]. High blood pressure in SHR may result from sympathetic hyperactivity and sympathetic vasoconstriction [20, 21]. In SHR, the femoral artery, a conductance medium-size artery, exhibits increased vasoconstriction to the sympathetic vasoconstrictor noradrenaline [22–24] and endothelial dysfunction in adulthood [25]. However, opposite findings were also described in the SHR femoral artery [26]. Nevertheless, pharmacological studies using the femoral artery are highly relevant for a better understanding of the pathophysiology of peripheral artery disease (PAD), whose incidence has an increasing tendency in the world population [27, 28] similar to hypertension. Many factors, including hypertension and advancing age, have been implicated in the pathogenesis of PAD [27, 29, 30]. Although the femoral arteries are not the main factor contributing to elevated peripheral resistance in hypertension, altered vascular function in early periods of life may be involved in various diseased states in old age. In addition, different mechanisms may be involved in the vascular aging in the aorta, the common femoral artery [31], and small mesenteric arteries [5] in various periods of life.

In order to determine vascular changes in early periods of life, we investigated alterations in BP, NO production, and vascular function of the isolated femoral artery of juvenile and young adult male normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats. In addition, we determined NO-dependent and -independent components of endothelium-dependent relaxation to investigate possible compensatory mechanisms participating in the maintenance of normal vascular function in juvenescence.

2. Materials and Methods

2.1. Animals and Experimental Design. Male 7-week-old (juvenile) and 22-week-old (young adult) SHR and WKY rats were used (n = 8–10). All the rats used in the present study were born in our certified animal facility (Institute of Normal and Pathological Physiology SAS). The rats were housed in groups of five animals per cage, each strain separately, in an air-conditioned room at constant temperature (22–24°C) and humidity (45–60%) at a 12 : 12-h light/dark cycle (06 : 00–18 : 00 h lights on) and they were maintained on a standard pellet diet and tap water *ad libitum*. All procedures used were approved by the State Veterinary and Food Administration of the Slovak Republic.

Systolic blood pressure (SBP) and heart rate (HR) were determined noninvasively in conscious rats by the tail-cuff method at the end of the experiment as described previously [32]. Body weight (BW) was recorded at the same time. Seven- and 22-week-old rats were killed by decapitation after a brief CO_2 anesthesia. Wet weights of the left heart ventricle (LVW) were determined for calculation of their relative weights (LVW/body weight) to evaluate the degree of cardiac hypertrophy.

2.2. Assessment of Vascular Reactivity of the Femoral Artery by Wire Myograph. Immediately after decapitation, the femoral artery was carefully excised and cleaned of adipose or connective tissue. The arteries were then cut into segments (1.28 ± 0.04 mm long) and mounted as ring-shaped preparations in the Mulvany-Halpern style small vessel wire myograph chamber (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark) to determine the vascular reactivity during isometric conditions as described elsewhere [33]. The preparations were bathed in modified physiological salt solution (PSS) oxygenated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C [26, 32]. The composition of the PSS was (in mmol/L) NaCl 118.99, KCl 4.69, $NaHCO_3$ 25, $MgSO_4 \cdot 7H_2O$ 1.17, KH_2PO_4 1.18, $CaCl_2 \cdot 2H_2O$ 2.5, Na_2EDTA 0.03, and glucose 5.5 (pH 7.4). The normalization procedure, the calculations for normalized inner diameter, and the experimental protocol for the femoral artery were described previously [25, 32].

Briefly, the contractile response to 125 mmol/L KCl (PSS was changed to KPSS in which NaCl was exchanged for an equimolar concentration of KCl) for 2 min was first obtained on each arterial ring followed by washings with PSS. An equilibration period of 20 min was allowed between each series of experiments. After confirming a sufficient contractile response to KPSS, experiments with noradrenaline (10^{-5} mol/L) were started to obtain phasic and tonic contractile responses [24]. Since the arteries must be optimally precontracted to assess relaxation responses, a submaximal tone was induced with 10^{-6} mol/L serotonin (Ser) [15, 28]. This precontraction agent was then used for all subsequent relaxation studies. When the contraction of the femoral artery to Ser reached a steady state, increasing concentrations of the vasodilator acetylcholine (ACh, 0.001 to 10 μmol/L) were added in cumulative manner to perform endothelium-dependent concentration-response curves [34]

followed by rinsings. To examine whether NO was involved in ACh-induced vasorelaxation of the femoral artery, N^G-nitro-L-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor, was added to the PSS at 300 μmol/L and was allowed to incubate for 25 min. In the presence of L-NAME, when ACh-induced NO release could be precluded, the concentration-response curve for ACh was repeated. The drugs were then washed out (PSS, 30 min) and the nitric oxide donor sodium nitroprusside (SNP, 0.001 to 10 μmol/L) was added in a cumulative fashion to assess an endothelium-independent, however NO-dependent, vascular relaxation. After the following wash-out, the femoral artery rings were stimulated again with high concentration of K^+ (125 mmol/L) in depolarising solution to induce maximal contraction (PSS was changed to KPSS) and then left to achieve a plateau. The maximal tension reached with this depolarizing solution was set as 100% to express the active tension generated by noradrenaline [35].

NO-mediated relaxation was determined by measuring the portion of ACh-induced relaxation that was abolished by L-NAME [36, 37]. Calculations were performed by determination of the area under the curve (AUC, in arbitrary units, au) of individual dose-response curves. The NO-mediated response was then calculated as the difference between the AUC of ACh-induced relaxation in the absence and presence of L-NAME. The extent of vasorelaxation was expressed in relative values as the percentage of the initial contraction induced by Ser as well as in absolute values (mN/mm) to minimize a possible effect of different Ser-induced precontraction tone [25, 38, 39]. Vasoconstrictions were determined as the maximal tension and they were expressed as active wall tension in mN/mm. All chemicals used were purchased from Sigma-Aldrich (Germany) and Merck Chemicals (Germany), except noradrenaline hydrogenotartras (Zentiva, Czech Republic).

2.3. Nitric Oxide Synthase Activity. Total NO synthase (NOS) activity was measured in tissue homogenates of the aorta (200 mg/mL) by determination of [^{3}H]-L-citrulline formation from [^{3}H]-L-arginine (MP Biomedicals, USA, 50 Ci/mmol), as described previously [32] and expressed as pmol/min/mg of tissue proteins as determined by the Lowry method [40]. All chemicals used were purchased from Sigma-Aldrich (Germany) and Merck Chemicals (Germany).

2.4. Statistical Analysis. Data are presented as group mean values ± SEM of the number (n) of independent measurements. Results were analyzed by analysis of variance (ANOVA). Two-way ANOVA (with age and strain as independent variables) was used to compare basic biometric and cardiovascular parameters and normalized inner diameter, vascular constrictions, and nitric oxide synthase activity. In case of significant results pairwaise comparison with Bonferroni adjustment was employed. Homogeneity of variances and normality of distribution were tested by Levene's test and by Shapiro-Wilk's test, respectively. Concentration response curves were compared using two-way repeated measurements ANOVA, followed by vertical contrast with Bonferroni adjustment. To assess depression present at high concentration of ACh-cumulative concentration response curves, the maximal response, and the response at higher ACh concentration at a particular response curve was compared with Dunnett's test. Means were considered to differ significantly when $P < 0.05$.

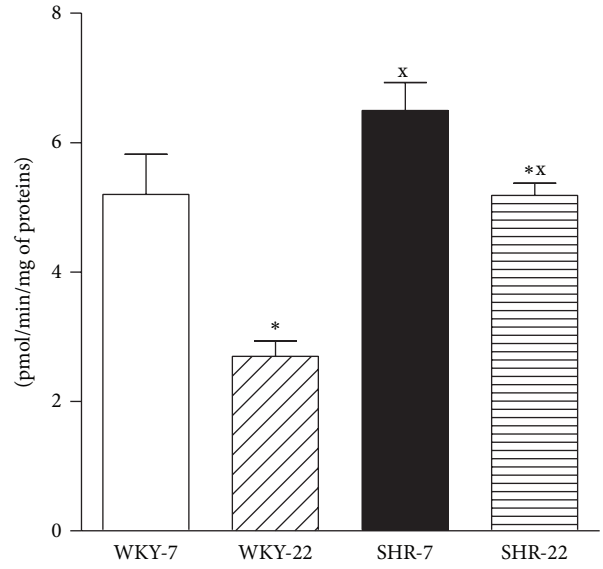

Figure 1: Nitric oxide synthase activity in the aorta of young adult (22-week-old) and juvenile (7-week-old) Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). SHR-7: 7-week-old SHR; SHR-22: 22-week-old SHR; WKY-7: 7-week-old WKY rats; WKY-22: 22-week-old WKY rats. Values represent mean ± SEM of 6–8 rats. Symbols have the following meanings: $^xP < 0.05$ compared to age-matched WKY (SHR-7 versus WKY-7, SHR-22 versus WKY-22), $^*P < 0.05$ compared to juvenile rats (WKY-22 versus WKY-7, SHR-22 versus SHR-7).

3. Results

Systolic blood pressure, heart rate, body weight, LVM/BW ratio, and vascular parameters of experimental groups are shown in Table 1. SBP, heart rate, and LVW/BW were significantly increased and BW and normalized inner diameter of the femoral artery was significantly decreased in both SHR groups as compared to age-matched WKY (Table 1). LVW/BW was lower in all young adult (22-week-old) groups than in juvenile rats. A significant age-dependent increase of BW and normalized inner diameter of the femoral artery was found in 22-week-old WKY and SHR; however age-related SBP increase was present only in 22-week-old SHR compared to juvenile 7-week-old SHR.

NOS activity was increased in SHR compared to age-matched WKY (Figure 1). Additionally, there were significant age-related decreases in NOS activity in the aorta of WKY and SHR (Figure 1).

ACh (1 nmol/L–10 μmol/L) and SNP (1 nmol/L–10 μmol/L) relaxed the femoral artery from both WKY and SHR in a concentration-dependent manner (Figure 2).

Table 1: Age-related effect on basic biometric and vascular parameters of the femoral artery of juvenile and young adult Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR).

	WKY		SHR	
	7 weeks	22 weeks	7 weeks	22 weeks
BW (g)	168 ± 8	416 ± 12*	135 ± 4^{x}	382 ± 8^{*x}
BP (mmHg)	114 ± 2	106 ± 2	159 ± 4^{x}	191 ± 3^{*x}
HR (bpm)	419 ± 13	405 ± 14	545 ± 10^{x}	521 ± 19^{x}
LVW/BW (mg/g)	1.99 ± 0.03	1.44 ± 0.03*	2.35 ± 0.06^{x}	2.18 ± 0.05^{*x}
ND (μm)	649 ± 17	769 ± 12*	556 ± 20^{x}	690 ± 13^{*x}
Ser (mN/mm)	5.63 ± 0.25	7.41 ± 0.15*	5.90 ± 0.40	6.05 ± 0.50^{x}
$\text{Ser}_{\text{L-NAME}}$ (mN/mm)	7.03 ± 0.45†	8.94 ± 0.31*†	7.44 ± 0.38†	7.80 ± 0.47†
$E_{\text{max ACh}}$ (mN/mm)	4.50 ± 0.21	4.80 ± 0.15	3.40 ± 0.25^{x}	2.83 ± 0.19^{x}
$E_{\text{max L-NAME}}$ (mN/mm)	3.38 ± 0.25†	4.54 ± 0.36*	1.57 ± 0.20^{x†}	1.99 ± 0.40^{x†}
$E_{\text{max SNP}}$ (%)	95.3 ± 0.9	93.9 ± 1.0	98.6 ± 0.8	94.0 ± 0.8
KPSS (mN/mm)	6.54 ± 0.61	9.17 ± 0.90*	7.42 ± 0.63	9.78 ± 0.48*

ACh: acetylcholine; BW: body weight; BP: blood pressure; $E_{\text{max ACh}}$: maximal acetylcholine-induced relaxation based on individual concentration-response curves; $E_{\text{max L-NAME}}$: maximal acetylcholine-induced relaxation after L-NAME based on individual concentration-response curves; $E_{\text{max SNP}}$: maximal sodium nitroprusside-induced relaxation based on individual concentration-response curves; HR: heart rate; KPSS: high-potassium physiological salt solution; L-NAME: N^{G}-nitro-L-arginine methyl ester; LVW: left heart ventricle weight; ND: normalized diameter of the femoral artery at 13.3 kPa calculated within the normalization procedure; Ser: vascular constriction induced by serotonin (1 μmol/L); $\text{Ser}_{\text{L-NAME}}$: vascular constriction induced by serotonin (1 μmol/L) after L-NAME; SNP: sodium nitroprusside. Results are mean ± S.E.M. of 6–10 rats. $^{x}P < 0.05$ compared to age-matched WKY (SHR-7 versus WKY-7, SHR-22 versus WKY-22), $^{*}P < 0.05$ compared to juvenile rats (WKY-22 versus WKY-7, SHR-22 versus SHR-7), and $^{\dagger}P < 0.05$ compared to the respective value before L-NAME.

Application of L-NAME abolished partially the effect of ACh, as illustrated in Figure 3. Since serotonin (1 μmol/L) induced different responses in 7- compared to 22-week-old WKY arteries and also in 22-week-old WKY compared to SHR femoral arteries resulting in a different prerelaxation active tension level (Table 1), the relaxation results were quantitatively expressed as mN/mm. However, calculations for relaxation responses in percentages (the percent of relaxation calculated relative to the steady-state contraction to Ser) revealed similar differences between the experimental groups and the results correlated well with the extent of prerelaxation active tone (data not shown). ACh-induced concentration-response curves were comparable in juvenile and young adult rats (Figure 2(a)). ACh-induced vasorelaxation was lower in SHR than that in age-matched WKY (Figure 2(a)). In SHR, maximal relaxation was achieved at ACh concentration of $3{\cdot}10^{-7}$ mol/L and a slight contractile effect counteracted the relaxant response at higher concentrations of ACh, resulting in a significant decrease in relaxation response at 10^{-5} mol/L ACh compared to the maximum relaxation response (Figure 2(a)). Cumulative addition of the NO donor sodium nitroprusside (SNP) produced similar relaxation responses in the femoral arteries from all experimental groups (Figure 2(b)).

L-NAME attenuated ACh-induced vasorelaxation in all groups investigated (Figure 3). The effect of L-NAME on ACh-induced relaxation did not differ between 7-week-old and 22-week-old SHR as compared to age-matched WKY (Figures 3 and 5). However, L-NAME attenuated ACh-induced relaxation in a smaller degree in young adult 22-week-old rats in both strains as compared to juvenile 7-week-old rats (Figures 3 and 5). When the arteries were pretreated with L-NAME, the femoral arteries from 7- and 22-week-old SHR rats responded by smaller relaxation to ACh than did the age-matched normotensive WKY arteries (Figures 4(c) and 4(d)), yet the femoral arteries from young adult 22-week-old rats responded in an even higher extent than did arteries from juvenile 7-week-old rats (Figures 4(a) and 4(b)).

NA-induced responses were biphasic: a transient contraction (early response, phasic contraction), which occurred within the first 10–15 sec and returned nearly to baseline was followed by sustained contraction (delayed response, tonic contraction), which reached steady maximum levels at 5 to 20 min. The tonic response of the 7- and 22-week-old SHR femoral artery to noradrenaline was greater than that in the age-matched WKY (Figure 6(a)). Phasic response was augmented in the 22-week-old SHR compared to the age-matched WKY. Aging significantly potentiated the contractions induced by NA in the femoral artery of both SHR and WKY. Unlike the NA-induced contraction, the KCl-induced contraction of the femoral artery seems to be unaffected in hypertensive rats, though an age-related increase was observed both in WKY and SHR (Table 1). Consequently, the ratio between the two contractile agents (NA/KCl) was still higher in the femoral artery of SHR as compared to that of WKY (Figure 6(b)). However only the tonic contraction induced by NA determined in relative values (calculated as percentage of maximal response induced by KPSS) was significantly greater in SHR than in WKY rats.

4. Discussion

The possible role of aging affecting vascular responses was studied in relation to experimental hypertension. In this study, we investigated age-related alterations in

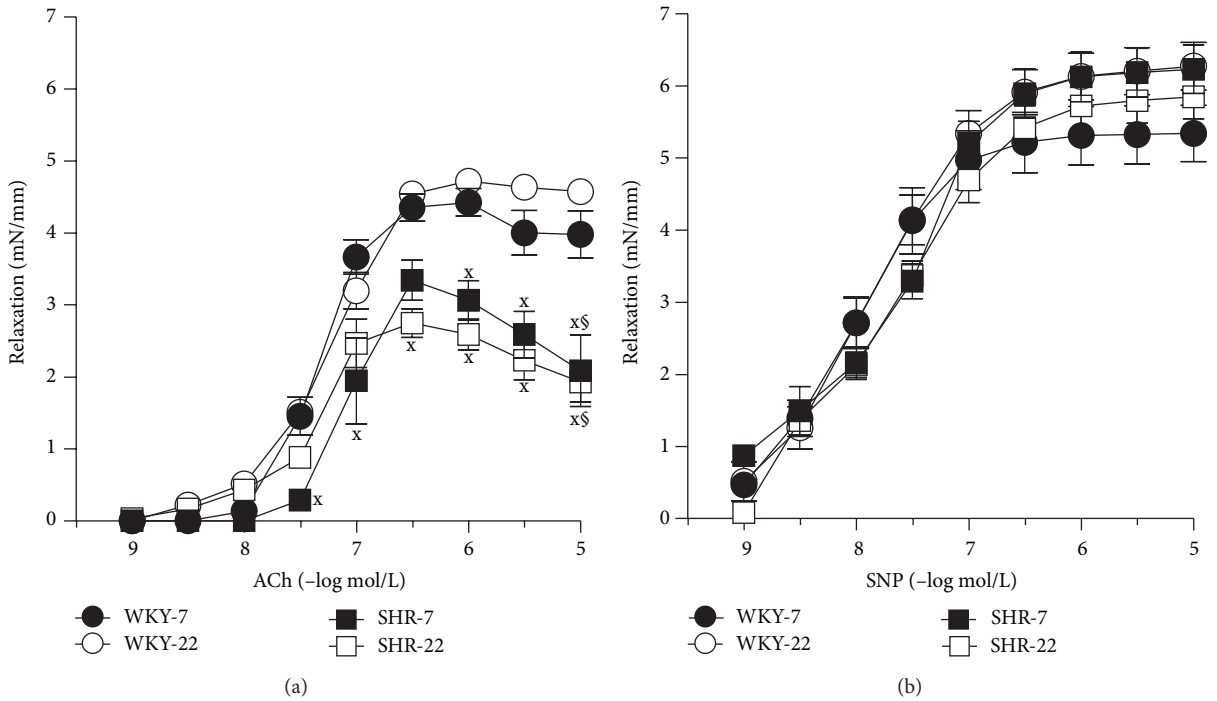

Figure 2: (a) Vascular responses to acetylcholine (ACh) and (b) sodium nitroprusside (SNP) in isolated femoral arteries of 7- and 22-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). Values represent mean ± SEM of 6–8 rats. SHR-7: 7-week-old SHR; SHR-22: 22-week-old SHR; WKY-7: 7-week-old WKY rats; WKY-22: 22-week-old WKY rats. Symbols have the following meanings: $^{x}P < 0.05$ compared to age-matched WKY (SHR-7 versus WKY-7, SHR-22 versus WKY-22), $^{\S}P < 0.05$, compared to maximal relaxation at ACh concentrations 0.3 μmol/L—this significance indicates the release of counterbalancing contracting factors in hypertensive animals.

acetylcholine-induced relaxation of the femoral artery, its L-NAME-sensitive and -resistant components, and total nitric oxide synthase activity in the aortas of juvenile and young adult WKY and SHR. The present study showed that (1) SBP was augmented in 7- and 22-week-old SHR compared to age-matched WKY and age-related augmentation of SBP was seen in SHR but not in WKY; (2) ACh-induced vasorelaxation in the femoral artery in 7-week-old rats did not differ from that in 22-week-old rats in either strain investigated; (3) ACh-induced relaxation in 7- and 22-week-old SHR was attenuated compared with that in age-matched WKY and this endothelial dysfunction originated from reduced NO-independent mechanisms and/or elevated release of EDCF, yet not from NO deficiency; (4) ACh-induced relaxation mediated by NO and NOS activity were attenuated in young adult (22-week-old) WKY and SHR compared with that in juvenile (7-week-old) rats; (5) NA-induced vasoconstriction was augmented in 7- and 22-week-old SHR compared to age-matched WKY; and finally, (6) aging augmented the responses to NA in both WKY and SHR.

In this study we determined NO synthase activity in the aorta and the L-NAME-sensitive component of ACh-induced relaxation in the femoral artery as measures of NO production and bioavailability in the vasculature. Although we could not measure NOS activity in the femoral arteries (due to insufficient amount of tissue for this method), it is assumed that, despite anatomic heterogeneity of the aorta and femoral artery [31], changes in NOS activity in the aorta correspond to those in NO-dependent relaxation of the femoral artery, as this association was shown previously in rats with blunted NO production [37]. Additionally, it is well known that acetylcholine-induced endothelium-dependent relaxation involves besides NO also other endothelium-derived relaxing factors. We therefore investigated vascular reactivity also in the presence of the nonspecific NOS inhibitor L-NAME, in order to assess NO-dependent (i.e., L-NAME-sensitive) and NO-independent (i.e., L-NAME-resistant) relaxation. Thus changes in NO production along with changes in the NO-dependent component of ACh-induced relaxation are clear indicators of NO bioavailability in the given artery.

Using this experimental approach, we observed that aging (in a relatively early period of life) reduced the total activity of NOS in the aorta and NO-dependent vasorelaxation of the femoral artery in both WKY and SHR. These alterations were however not associated with quantitatively blunted endothelium-dependent relaxations, as determined by the ACh test.

Data from the literature suggest that aging alters vascular function. Several studies have indicated that endothelium-dependent relaxations might decline with age. Aging was found to blunt NO-dependent relaxations in the mesenteric artery of male Wistar rats [8], in the aorta of healthy normotensive male and female rats [13, 14], in the common carotid artery of normotensive WKY and spontaneously hypertensive male rats [15], and in the coronary arterioles

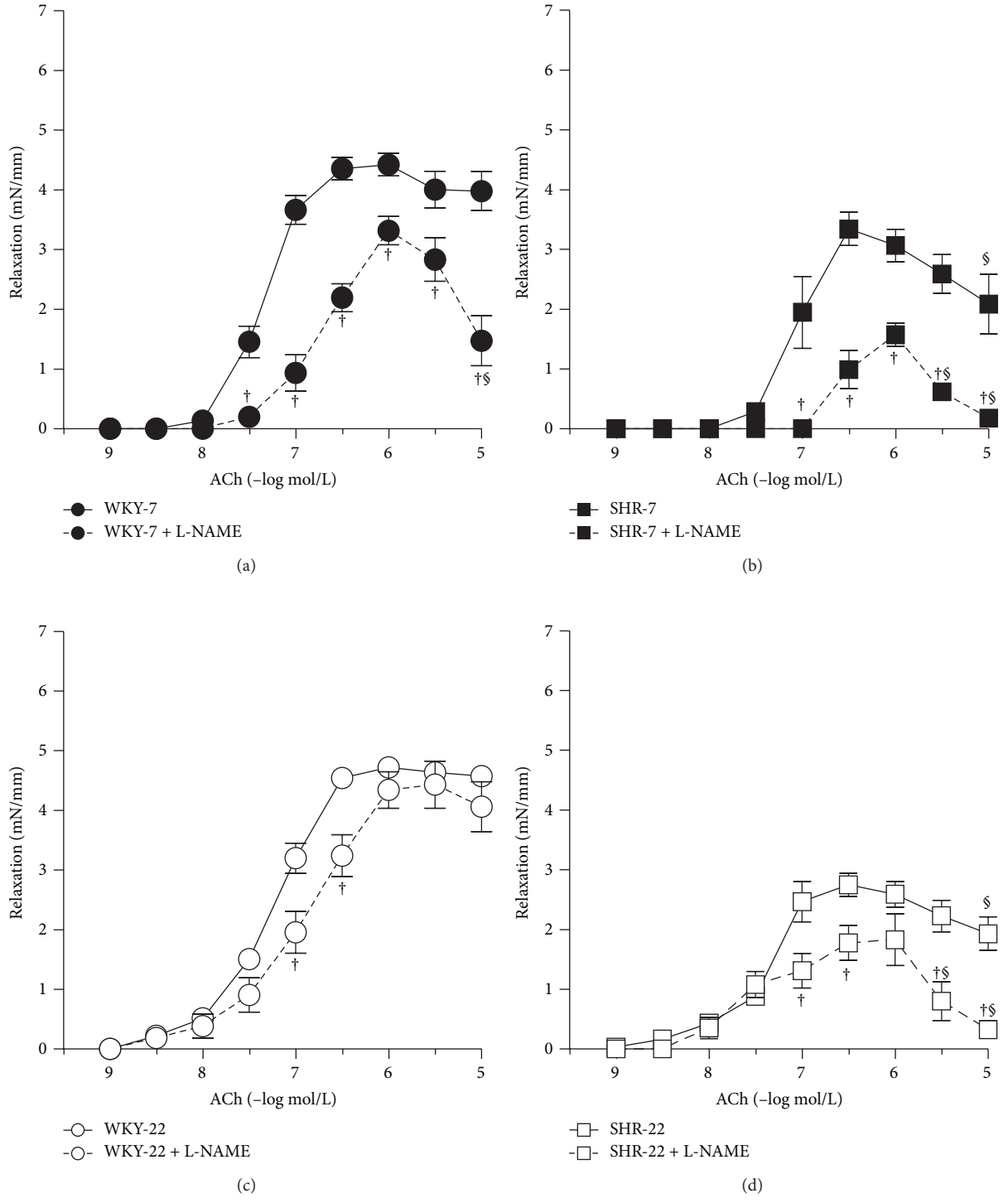

Figure 3: Vascular responses to acetylcholine (ACh) in isolated femoral arteries of 7- and 22-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) before (full lines) and after (dashed lines) incubation with the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L). Endothelium-dependent relaxations in the absence and presence of L-NAME of (a) 7-week-old WKY, (b) 7-week-old SHR, (c) 22-week-old WKY, and (d) 22-week-old SHR. Values represent mean ± SEM of 6–8 rats. SHR-7: 7-week-old SHR; SHR-22: 22-week-old SHR; WKY-7: 7-week-old WKY rats; WKY-22: 22-week-old WKY rats. Symbols have the following meanings: $^{\dagger}P < 0.05$ compared to the respective value before L-NAME; $^{\S}P < 0.05$, compared to maximal relaxation at ACh concentrations 0.3 or 1 μmol/L (a, c, d)—significance indicating release of counterbalancing vasocontractile factors in hypertensive animals and in L-NAME-treated femoral arteries from juvenile WKY and juvenile and young adult SHR.

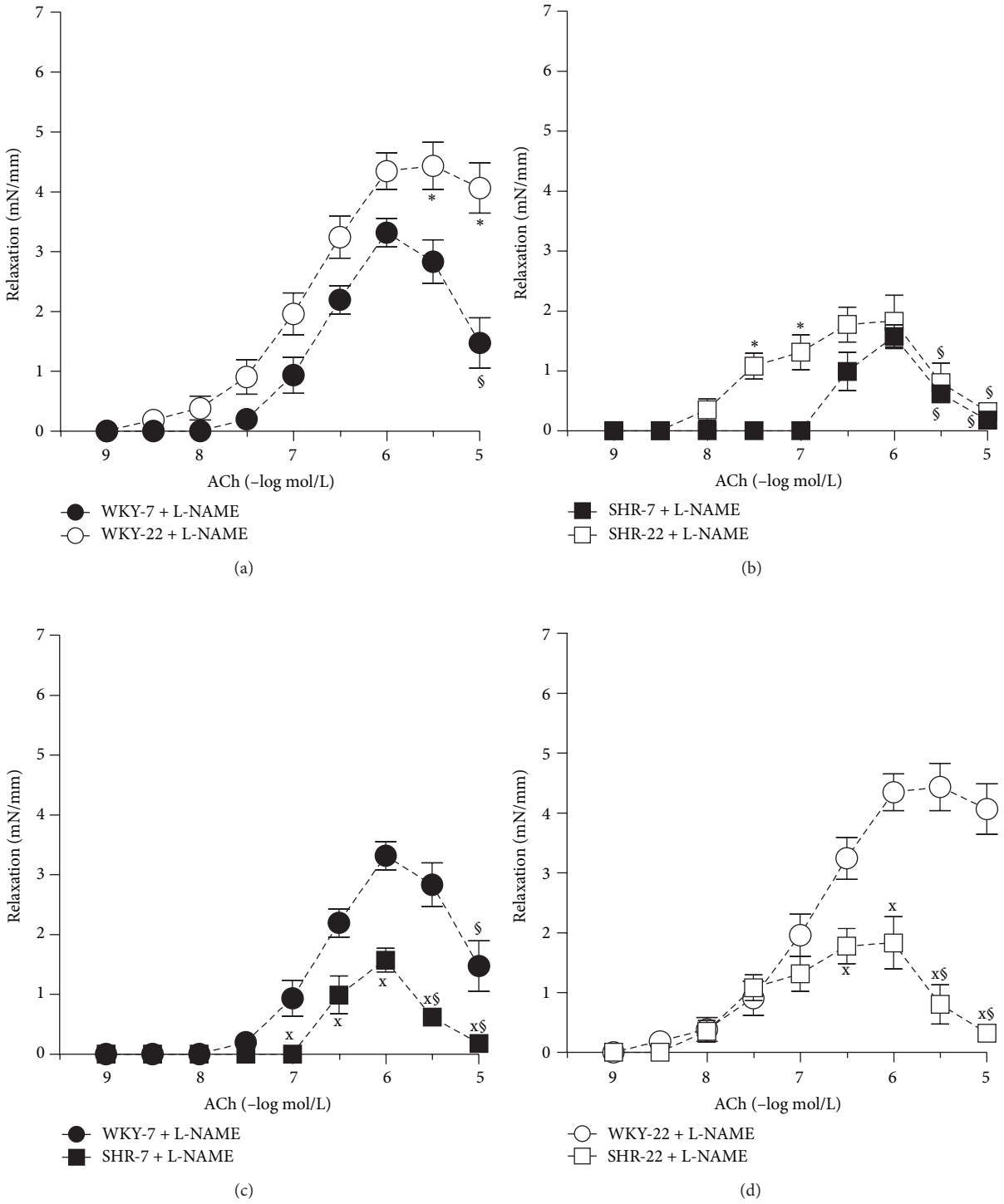

Figure 4: Vascular responses to acetylcholine (ACh) in isolated femoral arteries of 7- and 22-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) in the presence of nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L). Age-related effect on endothelium-dependent L-NAME-resistant relaxations in (a) 7- and 22-week-old WKY and (b) 7- and 22-week-old SHR. Differences between normotensive and hypertensive rats in (c) 7-week-old and (d) 22-week-old rats. Values represent mean ± SEM of 6–8 rats. SHR-7: 7-week-old SHR; SHR-22: 22-week-old SHR; WKY-7: 7-week-old WKY rats; WKY-22: 22-week-old WKY rats. Symbols have the following meanings: $^{x}P < 0.05$ compared to age-matched WKY (SHR-7 versus WKY-7, SHR-22 versus WKY-22), $^{*}P < 0.05$ compared to juvenile rats (WKY-22 versus WKY-7, SHR-22 versus SHR-7), and $^{\S}P < 0.05$ compared to maximal relaxation at ACh concentrations 0.3 or 1 μmol/L (a, b, c, d)—significance indicating release of counterbalancing vasocontractile factors in hypertensive animals and in L-NAME-treated femoral arteries from juvenile WKY and juvenile and young adult SHR.

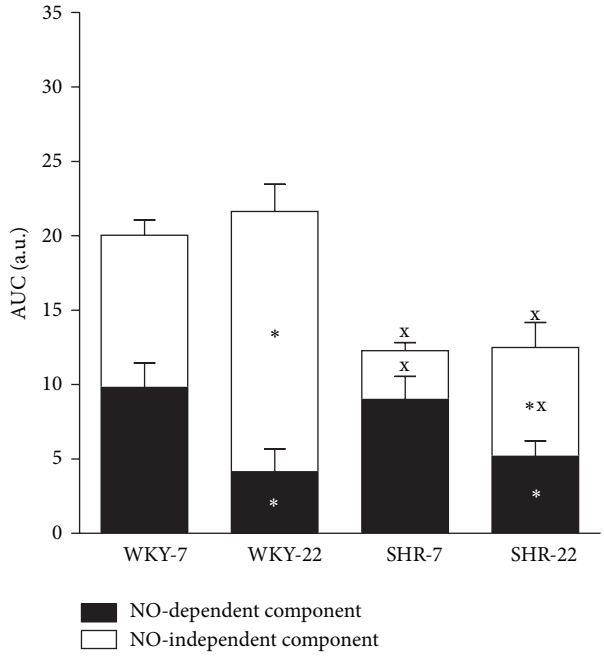

Figure 5: Area under the curve (AUC) of the vascular responses, based on the individual concentration-dependent relaxation curves to acetylcholine (ACh) in isolated femoral arteries of 7- and 22-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). Values represent mean ± SEM of 6–8 rats. SHR-7: 7-week-old SHR; SHR-22: 22-week-old SHR; WKY-7: 7-week-old WKY rats; WKY-22: 22-week-old WKY rats. Marks of significances over the entire columns represent significant differences between the total ACh-induced relaxations. Marks inside the black part indicate significances between NO-dependent component of vasorelaxation and marks inside the white bar represent the significant differences between the NO-independent components. Symbols have the following meanings: $^{x}P < 0.05$ compared to age-matched WKY (SHR-7 versus WKY-7, SHR-22 versus WKY-22), $^{*}P < 0.05$ compared to juvenile rats (WKY-22 versus WKY-7, SHR-22 versus SHR-7).

from Sprague-Dawley male rats [16], but it did not decrease relaxation in the pulmonary artery of normotensive female rats [14].

In this study, despite unchanged total ACh-induced relaxation, we observed significant qualitative age-related changes in the mechanism of relaxation by acute preincubation with L-NAME. NO-dependent components of the ACh-induced relaxation were reduced in young adult rats in association with reduced NOS activity in both strains investigated. Although we did not measure the activity of individual NOS isoforms, there are studies indicating the participation of eNOS and iNOS in age-related alterations [11]. In contrast, during a period of life comparable to that investigated in our study (4 weeks versus 14–17 weeks), reduction of eNOS activity was not observed in the aortas of WKY and SHR [41]. Chou et al. [41] found reduced activity of eNOS only in the aortas of WKY and that in much older rats (63-week-old). In addition, the same study showed that the basal activity and protein expression of iNOS were detected only in elderly (63-week-old) SHR and WKY and in adult (14-to-17-week old) SHR yet not in adult WKY, suggesting that the abnormal expression of iNOS is associated with hypertension. This abnormal iNOS expression might be implicated also in our study concerning the finding that SHR exhibited a significantly increased NOS activity than did age-matched WKY rats. Thus the observed increased total NOS activity in the aortas from SHR as compared to WKY at both ages studied may be related to the abnormal expression of iNOS or to a compensatory mechanism by eNOS activated in hypertension [42, 43]. However, elevated NOS expression need not be always associated with improved enzyme activity. Indeed, in the aortas from aged male Wistar rats, the expression of eNOS isoform was enhanced; however its activity was markedly reduced [44]. Reduced activity of NOS was also found in this study and may account for age-related decrease of NO-mediated vasorelaxation, independently of NO breakdown by reactive oxygen species [5].

Interestingly, we observed a significant age-related increase in the L-NAME-resistant (i.e., NO-independent) component of ACh-induced relaxation in WKY and SHR. This result is in contradistinction to previous reports which showed a rather decreased EDHF-mediated relaxation in vascular aging [8]. The disparity with our results may be related to differences in age and vascular bed of the animals studied. Although it is assumed that elevation of the NO-independent component of vasorelaxation in young adult rats (versus juvenile) was elicited by compensatory release of EDHF(s) and/or vasodilatory prostanoids, the effect of other endothelium derived factors cannot be ruled out.

Our findings also indicate that other than NO-mediated mechanisms can effectively be activated to compensate the loss of NO bioavailability in early vascular aging. This explanation has been suggested in the study of Sofola et al. [45] who indicated that EDHF might compensate the loss of NO and preserve the endothelium-dependent relaxation in the mesenteric arteries of hypertensive rats in which hypertension was induced by a high-salt diet. Moreover, compensatory EDHF production has been suggested to occur in normotensive rats after acute NOS inhibition [46]. In addition, the compensatory release of vasodilatory prostanoids may be involved [5, 47].

Nevertheless, our study showed that NO-independent endothelial dysfunction was present in SHR even at the age of 7 weeks. Similarly did Mori et al. [36] show that the EDHF-mediated responses were attenuated before the loss of NO-mediated dilatation in the femoral resistance arteries of SHR compared to WKY. Our results also indicated that the effect of L-NAME on vasorelaxation did not differ in WKY and age-matched SHR; thus there were no significant differences in NO-mediated responses. Similarly, in resistance femoral arteries, there were no differences in NO-mediated vasodilatation in 5- and 15-week-old SHR compared to WKY [36]. We also assume that the decrease of NO-independent relaxation of the SHR femoral artery may be related to a reduced EDHF-dependent component because PGI_2 inhibition by indomethacin failed to show any effects on ACh-induced relaxation of hindlimb arteries of SHR and WKY at various periods of life [36]. Yet as there is a complex cross-talk among individual EDRFs [10],

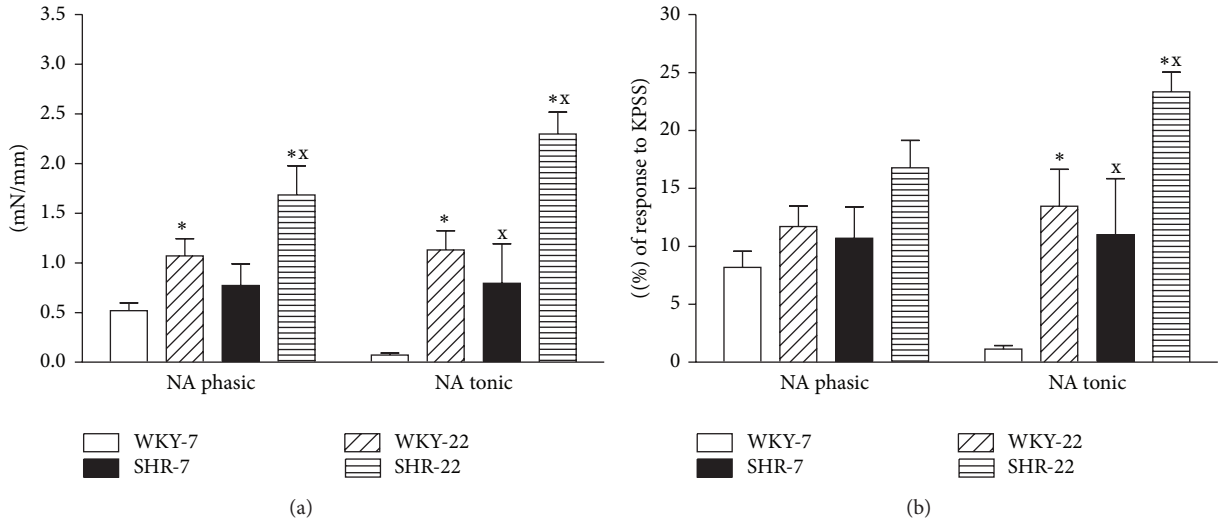

Figure 6: Vascular constrictions induced by noradrenaline (NA, 10 μmol/L) in the femoral artery of young adult (22-week-old) and juvenile (7-week-old) Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). SHR-7: 7-week-old SHR; SHR-22: 22-week-old SHR; WKY-7: 7-week-old WKY rats; WKY-22: 22-week-old WKY rats. Values represent mean ± SEM of 6–8 rats. Symbols have the following meanings: $^{x}P < 0.05$ compared to age-matched WKY (SHR-7 versus WKY-7, SHR-22 versus WKY-22), $^{*}P < 0.05$ compared to juvenile rats (WKY-22 versus WKY-7, SHR-22 versus SHR-7).

the contribution of individual EDRFs to alterations of NO-independent components of ACh-induced relaxation needs to be evaluated in specifically designed studies.

Regarding vasoconstriction, elevated responses to serotonin were observed in all groups investigated after NOS inhibition. As the active tone level was decreased (not increased) in young adult SHR and comparable in the arteries from juvenile rats, it is not assumed that the level of precontraction tone could account for the reduced endothelial relaxation in SHR as compared to age-matched WKY. We found an increase in NA-induced vasoconstriction in hypertensive rats compared to normotensive age-matched controls and aging augmented further the noradrenaline-induced contractions of femoral arteries in SHR. This finding is in contrast to the report of Konishi and Su [26] showing unaffected responses to NA in 15-16-week-old SHR femoral arteries. However, other investigators [22, 23] reported similar augmentations of NA responses in the femoral artery of 16–20-week-old male SHR compared to age-matched normotensive rats. A similar augmentation of NA-induced responses was found in the aorta of male SHR [48] and the femoral artery of adult female SHR [49]. Since KCl acts directly on smooth muscle cells [25], the augmented NA response observed in SHR, in relation to KCl, indicates alterations in NA receptors and/or signaling, alterations of Ca^{2+} handling and sensitivity [22, 50–52], and structural remodeling of the arteries. Furthermore, EDCF contribution to NA-induced contraction of femoral arteries was reported to be similarly enhanced in adult (7-month-old) SHR and aged (14-month-old) normotensive WKY rats [39]. In addition, endothelium-dependent contractions elicited by high concentrations of acetylcholine were described both in hypertensive and in aged normotensive rats [53–55]. Cyclooxygenase-dependent EDCF production was found to be characteristic of aging [5, 56]. Our findings also support the role of EDCF(s) in hypertension-related endothelial dysfunction in SHR (Figures 3(b) and 3(d)). Moreover, augmented EDCF(s)-induced decline in relaxation responses after L-NAME-pretreatment in both SHR groups and juvenile WKY suggests neutralization of EDCF(s) action by NO. This mechanism was reported previously by Auch-Schwelk et al. [57].

It has been widely accepted that aging and hypertension do not affect sensitivity of vascular smooth muscle to NO (e.g., relaxation induced by endothelium-independent vascular smooth muscle relaxants such as sodium nitroprusside) and yet some conflicting findings were reported [15, 58, 59]. In our experiments, SNP-induced concentration-dependent relaxation did not differ between the groups, indicating that a decrease in the NO-mediated ACh-induced relaxations was due to specific impairment of the endothelium-dependent mechanism rather than to changes in vascular smooth muscle cells. Several studies have suggested that reduced NO bioavailability during aging can be related to increased production of reactive oxygen species. Indeed, aging was associated with blunted endothelium-dependent relaxations and excessive vascular formation of reactive oxygen species in Wistar, SHR, and WKY rats [8, 60]. It should however be noted that the oxidative stress theory of aging has to be further elucidated due to significant animal-strain-related differences in the effect of reactive oxygen species [61, 62].

It is well known that elevated vascular resistance along with increased stiffness of conduit arteries participate in aging and in hypertension development [2, 63]. We found that SBP increased significantly with aging in SHR, which was not observed in WKY. Thus chronic exposure of the femoral artery to higher pressure may not be the primary cause

of age-related decrease of NO synthase inhibitor sensitive response to ACh. Moreover, our results suggest that age-related elevation of blood pressure between the 7th and 22nd week of life in SHR does not result from worsened endothelium-dependent relaxation but rather from elevated sympathetic vasoconstriction.

An elevated sympathetic tone in SHR was shown previously [19, 20] and our observation of increased heart rate in SHR at both ages studied is in agreement with these studies. Moreover, it has been reported that the hyperactivity of the sympathetic nervous system could induce structural and functional alterations of the heart and blood vessels in SHR [20, 64–66]. In addition, age-related decrease in the NO-dependent relaxation in the femoral artery along with its structural remodeling may be implicated in the pathogenesis of peripheral artery disease. Further studies are however needed to evaluate the underlying factors and the exact mechanism of age-related decrease in NO-dependent vasorelaxation in the femoral artery and their involvement in the pathogenesis of peripheral artery disease and hypertension.

5. Conclusions

In our study aging between the 7th and 22nd week of life was associated with decreased vascular NO production and NO-mediated vasorelaxation. However reduction in NO bioavailability did not result in endothelial dysfunction as the reduction of NO-dependent component of relaxation was fully compensated by accentuation of NO-independent relaxation in both WKY and SHR. The results suggest that NO-independent mechanisms can act as a salvage system to maintain endothelial function in situations associated with decreased NO bioavailability, at least in early periods of life. The exact role of NO in aging and hypertension remains however still open.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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References

[1] E. G. Lakatta and D. Levy, "Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises. Part I. Aging arteries: a "set up" for vascular disease," *Circulation*, vol. 107, no. 1, pp. 139–146, 2003.

[2] P. M. Nilsson, P. Boutouyrie, and S. Laurent, "Vascular aging: a tale of EVA and ADAM in cardiovascular risk assessment and prevention," *Hypertension*, vol. 54, no. 1, pp. 3–10, 2009.

[3] K. Egashira, T. Inou, Y. Hirooka et al., "Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans," *Circulation*, vol. 88, no. 1, pp. 77–81, 1993.

[4] D. S. Celermajer, K. E. Sorensen, D. J. Spiegelhalter, D. Georgakopoulos, J. Robinson, and J. E. Deanfield, "Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women," *Journal of the American College of Cardiology*, vol. 24, no. 2, pp. 471–476, 1994.

[5] R. L. Matz, M. A. de Sotomayor, C. Schott, J. C. Stoclet, and R. Andriantsitohaina, "Vascular bed heterogeneity in age-related endothelial dysfunction with respect to NO and eicosanoids," *British Journal of Pharmacology*, vol. 131, no. 2, pp. 303–311, 2000.

[6] R. L. Matz, C. Schott, J. C. Stoclet, and R. Andriantsitohaina, "Age-related endothelial dysfunction with respect to nitric oxide, endothelium-derived hyperpolarizing factor and cyclooxygenase products," *Physiological Research*, vol. 49, no. 1, pp. 11–18, 2000.

[7] S. Taddei, A. Virdis, L. Ghiadoni et al., "Age-related reduction of NO availability and oxidative stress in humans," *Hypertension*, vol. 38, no. 2, pp. 274–279, 2001.

[8] S. Dal-Ros, C. Bronner, C. Auger, and V. B. Schini-Kerth, "Red wine polyphenols improve an established aging-related endothelial dysfunction in the mesenteric artery of middle-aged rats: role of oxidative stress," *Biochemical and Biophysical Research Communications*, vol. 419, no. 2, pp. 381–387, 2012.

[9] M. Feletou and P. M. Vanhoutte, "Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture)," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 291, pp. 985–1002, 2006.

[10] I. Bernatova, "Endothelial dysfunction in experimental models of arterial hypertension: cause or consequence?" *BioMed Research International*, vol. 2014, Article ID 598271, 14 pages, 2014.

[11] H. J. Yoon, S. W. Cho, B. W. Ahn, and S. Y. Yang, "Alterations in the activity and expression of endothelial NO synthase in aged human endothelial cells," *Mechanisms of Ageing and Development*, vol. 131, no. 2, pp. 119–123, 2010.

[12] A. M. Briones, M. Salaices, and E. Vila, "Ageing alters the production of nitric oxide and prostanoids after IL-1β exposure in mesenteric resistance arteries," *Mechanisms of Ageing and Development*, vol. 126, no. 6-7, pp. 710–721, 2005.

[13] B. van der Loo, R. Labugger, J. N. Skepper et al., "Enhanced peroxynitrite formation is associated with vascular aging," *Journal of Experimental Medicine*, vol. 192, no. 12, pp. 1731–1743, 2000.

[14] M. R. Tschudi, M. Barton, N. A. Bersinger et al., "Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery," *Journal of Clinical Investigation*, vol. 98, no. 4, pp. 899–905, 1996.

[15] K. Hongo, T. Nakagomi, N. F. Kassell et al., "Effects of aging and hypertension on endothelium-dependent vascular relaxation in rat carotid artery," *Stroke*, vol. 19, no. 7, pp. 892–897, 1988.

[16] A. Csiszar, Z. Ungvari, J. G. Edwards et al., "Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function," *Circulation Research*, vol. 90, no. 11, pp. 1159–1166, 2002.

[17] Y. Yamori and K. Okamoto, "Spontaneous hypertension in rats versus essential hypertension in man," *Singapore Medical Journal*, vol. 14, no. 3, pp. 393–394, 1973.

[18] M. Pintérová, J. Kuneš, and J. Zicha, "Altered neural and vascular mechanisms in hypertension," *Physiological Research*, vol. 60, no. 3, pp. 381–402, 2011.

[19] J. Zicha and J. Kuneš, "Ontogenetic aspects of hypertension development: analysis in the rat," *Physiological Reviews*, vol. 79, no. 4, pp. 1227–1282, 1999.

[20] M. Behuliak, M. Pintérová, J. Kuneš, and J. Zicha, "Vasodilator efficiency of endogenous prostanoids, Ca^{2+}-activated K^+ channels and nitric oxide in rats with spontaneous, salt-dependent or NO-deficient hypertension," *Hypertension Research*, vol. 34, no. 8, pp. 968–975, 2011.

[21] A. Zemančíková and J. Török, "Effect of chronic nifedipine treatment on blood pressure and adrenergic responses of isolated mesenteric artery in young rats with developing spontaneous hypertension," *Physiological Research*, vol. 58, no. 6, pp. 921–925, 2009.

[22] S. Líšková, M. Petrová, P. Karen, J. Kuneš, and J. Zicha, "Influence of calcium-dependent potassium channel blockade and nitric oxide inhibition on norepinephrine-induced contractions in two forms of genetic hypertension," *Journal of the American Society of Hypertension*, vol. 4, no. 3, pp. 128–134, 2010.

[23] M. Petrova, S. Liskova, R. Vojtko et al., "Vascular reactivity of arteria femoralis in adult and aged spontaneously hypertensive and Wistar-Kyoto rats," *Bratislavske lekarske listy*, vol. 114, pp. 553–555, 2013.

[24] L. Paulis, S. Líšková, M. Pintérová, Z. Dobešová, J. Kuneš, and J. Zicha, "Nifedipine-sensitive noradrenergic vasoconstriction is enhanced in spontaneously hypertensive rats: the influence of chronic captopril treatment," *Acta Physiologica*, vol. 191, no. 4, pp. 255–266, 2007.

[25] A. Puzserova, J. Kopincova, P. Slezak, P. Balis, and I. Bernatova, "Endothelial dysfunction in femoral artery of the hypertensive rats is nitric oxide independent," *Physiological Research*, vol. 62, pp. 615–629, 2013.

[26] M. Konishi and C. Su, "Role of endothelium in dilator responses of spontaneously hypertensive rat arteries," *Hypertension*, vol. 5, no. 6, pp. 881–886, 1983.

[27] H. Suzuki and Y. Iso, "Clinical application of vascular regenerative therapy for peripheral artery disease," *BioMed Research International*, vol. 2013, Article ID 179730, 6 pages, 2013.

[28] M. A. Delbin, A. S. Silva, E. Antunes, and A. Zanesco, "Interaction between serotoninergic-and β-adrenergic receptors signaling pathways in rat femoral artery," *Arquivos Brasileiros de Cardiologia*, vol. 98, no. 1, pp. 29–34, 2012.

[29] M. M. Joosten, J. K. Pai, M. L. Bertoia et al., "Associations between conventional cardiovascular risk factors and risk of peripheral artery disease in men," *Journal of the American Medical Association*, vol. 308, pp. 1660–1667, 2012.

[30] C. Diehm, S. Kareem, and H. Lawall, "Epidemiology of peripheral arterial disease," *Journal of Vascular Diseases*, vol. 33, no. 4, pp. 183–189, 2004.

[31] M. Barton, F. Cosentino, R. P. Brandes, P. Moreau, S. Shaw, and T. F. Lüscher, "Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin," *Hypertension*, vol. 30, no. 4, pp. 817–824, 1997.

[32] A. Puzserova, P. Slezak, P. Balis, and I. Bernatova, "Long-term social stress induces nitric oxide-independent endothelial dysfunction in normotensive rats," *Stress*, vol. 16, pp. 331–339, 2013.

[33] M. J. Mulvany and W. Halpern, "Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats," *Circulation Research*, vol. 41, no. 1, pp. 19–26, 1977.

[34] S. Fujimoto, K. S. Fujimoto, and A. Moriyama, "Diminished β-adrenoceptor-mediated relaxation of femoral arteries from young spontaneously hypertensive rats," *Autonomic Neuroscience*, vol. 87, no. 2-3, pp. 178–186, 2001.

[35] A. Puzserova and I. Bernatova, "Chronic social stress increases nitric oxide-dependent vasorelaxation in normotensive rats," *Interdisciplinary Toxicology*, vol. 3, no. 4, pp. 109–117, 2010.

[36] Y. Mori, M. Ohyanagi, S. Koida, A. Ueda, K. Ishiko, and T. Iwasaki, "Effects of endothelium-derived hyperpolariziing factor and nitric oxide on endothelial function in femoral resistance arteries of spontaneously hypertensive rats," *Hypertension Research*, vol. 29, no. 3, pp. 187–195, 2006.

[37] L. Paulis, J. Zicha, J. Kunes et al., "Regression of L-NAME-induced hypertension: the role of nitric oxide and endothelium-derived constricting factor," *Hypertension Research*, vol. 31, no. 4, pp. 793–803, 2008.

[38] K. Hansen and O. A. Nedergaard, "Methodologic aspects of acetylcholine-evoked relaxation of rabbit aorta," *Journal of Pharmacological and Toxicological Methods*, vol. 41, no. 4, pp. 153–159, 1999.

[39] S. Líšková, M. Petrová, P. Karen, J. Kuneš, and J. Zicha, "Effects of aging and hypertension on the participation of endothelium-derived constricting factor (EDCF) in norepinephrine-induced contraction of rat femoral artery," *European Journal of Pharmacology*, vol. 667, no. 1–3, pp. 265–270, 2011.

[40] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *The Journal of biological chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[41] T. C. Chou, M. H. Yen, C. Y. Li, and Y. A. Ding, "Alterations of nitric oxide synthase expression with aging and hypertension in rats," *Hypertension*, vol. 31, no. 2, pp. 643–648, 1998.

[42] O. Pechanova, J. Zicha, S. Kojsova, Z. Dobesova, L. Jendekova, and J. Kunes, "Effect of chronic N-acetylcysteine treatment on the development of spontaneous hypertension," *Clinical Science*, vol. 110, pp. 235–242, 2006.

[43] J. Török, "Participation of nitric oxide in different models of experimental hypertension," *Physiological Research*, vol. 57, no. 6, pp. 813–825, 2008.

[44] M. R. Cernadas, L. S. de Miguel, M. García-Durán et al., "Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats," *Circulation Research*, vol. 83, no. 3, pp. 279–286, 1998.

[45] O. A. Sofola, A. Knill, R. Hainsworth, and M. Drinkhill, "Change in endothelial function in mesenteric arteries of Sprague-Dawley rats fed a high salt diet," *Journal of Physiology*, vol. 543, no. 1, pp. 255–260, 2002.

[46] J. Bauersachs, R. Popp, M. Hecker, E. Sauer, I. Fleming, and R. Busse, "Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor," *Circulation*, vol. 94, no. 12, pp. 3341–3347, 1996.

[47] D. Henrion, E. Dechaux, F. J. Dowell et al., "Alteration of flow-induced dilatation in mesenteric resistance arteries of L-NAME treated rats and its partial association with induction of cyclo-oxygenase-2," *British Journal of Pharmacology*, vol. 121, no. 1, pp. 83–90, 1997.

[48] S. Arribas, J. Marin, A. Ponte, G. Balfagon, and M. Salaices, "Norepinephrine-induced relaxations in rat aorta mediated by endothelial beta adrenoceptors. Impairment by ageing and hypertension," *Journal of Pharmacology and Experimental Therapeutics*, vol. 270, no. 2, pp. 520–527, 1994.

[49] M. Asano, K. Aoki, and T. Matsuda, "Reduced beta adrenoreceptor interactions of norepinephrine enhance contraction in the femoral artery from spontaneously hypertensive rats,"

Journal of Pharmacology and Experimental Therapeutics, vol. 223, no. 1, pp. 207–214, 1982.

[50] G. J. Lagaud, V. Randriamboavonjy, G. Roul, J. C. Stoclet, and R. Andriantsitohaina, "Mechanism of Ca^{2+} release and entry during contraction elicited by norepinephrine in rat resistance arteries," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 276, no. 1, pp. H300–H308, 1999.

[51] F. P. Field and E. E. Soltis, "Vascular reactivity in the spontaneously hypertensive rat: effect of high pressure stress and extracellular calcium," *Hypertension*, vol. 7, no. 2, pp. 228–235, 1985.

[52] M. S. Bal, L. Paulis, J. Zicha, and J. Kuneš, "Effect of protein kinase C and protein kinase A inhibitors on contraction of isolated femoral arteries of SHR and Wistar rats," *Physiological Research*, vol. 58, no. 6, pp. 793–798, 2009.

[53] P. M. Vanhoutte, "Endothelial dysfunction—The first step toward coronary arteriosclerosis," *Circulation Journal*, vol. 73, no. 4, pp. 595–601, 2009.

[54] M. Félétou, T. J. Verbeuren, and P. M. Vanhoutte, "Endothelium-dependent contractions in SHR: a tale of prostanoid TP and IP receptors," *British Journal of Pharmacology*, vol. 156, no. 4, pp. 563–574, 2009.

[55] M. S. K. Wong and P. M. Vanhoutte, "COX-mediated endothelium-dependent contractions: from the past to recent discoveries," *Acta Pharmacologica Sinica*, vol. 31, no. 9, pp. 1095–1102, 2010.

[56] D. Versari, E. Daghini, A. Virdis, L. Ghiadoni, and S. Taddei, "Endothelium-dependent contractions and endothelial dysfunction in human hypertension," *British Journal of Pharmacology*, vol. 157, no. 4, pp. 527–536, 2009.

[57] W. Auch-Schwelk, Z. S. Katusic, and P. M. Vanhoutte, "Nitric oxide inactivates endothelium-derived contracting factor in the rat aorta," *Hypertension*, vol. 19, no. 5, pp. 442–445, 1992.

[58] H. Wuorela, P. Arvola, M. Kahonen, H. Vapaatalo, and I. Porsti, "Arterial smooth muscle responses in adult and moderately aged spontaneously hypertensive rats," *Pharmacology and Toxicology*, vol. 74, no. 3, pp. 167–173, 1994.

[59] S. Fujimoto, Y. Dohi, K. Aoki, M. Asano, and T. Matsuda, "Diminished β-adrenoceptor-mediated relaxation of arteries from spontaneously hypertensive rats before and during development of hypertension," *European Journal of Pharmacology*, vol. 136, no. 2, pp. 179–187, 1987.

[60] S. Simão, P. Gomes, V. Pinto et al., "Age-related changes in renal expression of oxidant and antioxidant enzymes and oxidative stress markers in male SHR and WKY rats," *Experimental Gerontology*, vol. 46, no. 6, pp. 468–474, 2011.

[61] R. Buffenstein, Y. H. Edrey, T. Yang, and J. Mele, "The oxidative stress theory of aging: embattled or invincible? Insights from non-traditional model organisms," *Age*, vol. 30, no. 2-3, pp. 99–109, 2008.

[62] V. I. Pérez, A. Bokov, H. V. Remmen et al., "Is the oxidative stress theory of aging dead?" *Biochimica et Biophysica Acta*, vol. 1790, no. 10, pp. 1005–1014, 2009.

[63] S. M. Arribas, A. M. Briones, C. Bellingham et al., "Heightened aberrant deposition of hard-wearing elastin in conduit arteries of prehypertensive SHR is associated with increased stiffness and inward remodeling," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 295, no. 6, pp. H2299–H2307, 2008.

[64] M. Cebova and F. Kristek, "Age-dependent ultrastructural changes of coronary artery in spontaneously hypertensive rats," *General Physiology and Biophysics*, vol. 30, no. 4, pp. 364–372, 2011.

[65] M. Gerová and F. Kristek, "Integrative pattern of vasomotor efficiency in SHR during ontogenesis," *Physiological Research*, vol. 57, no. 5, pp. 677–684, 2008.

[66] A. Zemancíková and J. Török, "Diminished contractile responses of isolated conduit arteries in two rat models of hypertension," *The Chinese Journal of Physiology*, vol. 56, no. 4, pp. 230–235, 2013.

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Review Article

Recent Insights in the Paracrine Modulation of Cardiomyocyte Contractility by Cardiac Endothelial Cells

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The cardiac endothelium is formed by a continuous monolayer of cells that line the cavity of the heart (endocardial endothelial cells (EECs)) and the luminal surface of the myocardial blood vessels (intramyocardial capillary endothelial cells (IMCEs)). EECs and IMCEs can exercise substantial control over the contractility of cardiomyocytes by releasing various factors such as nitric oxide (NO) *via* a constitutive endothelial NO-synthase (eNOS), endothelin-1, prostaglandins, angiotensin II, peptide growth factors, and neuregulin-1. The purpose of the present paper is actually to shortly review recent new information concerning cardiomyocytes as effectors of endothelium paracrine signaling, focusing particularly on contractile function. The modes of action and the regulatory paracrine role of the main mediators delivered by cardiac endothelial cells upon cardiac contractility identified in cardiomyocytes are complex and not fully described. Thus, careful evaluation of new therapeutic approaches is required targeting important physiological signaling pathways, some of which have been until recently considered as deleterious, like reactive oxygen species. Future works in the field of cardiac endothelial cells and cardiac function will help to better understand the implication of these mediators in cardiac physiopathology.

1. Introduction

The purpose of the present review is actually to shortly review recent new information concerning cardiomyocytes as effectors of endothelium paracrine signaling, focusing particularly on contractile function. For more information on the cardiac endothelial modulating factors and their roles in the regulation of other heart functions (growth, differentiation, rhythmicity, remodeling), please refer to some recent reviews [1–5].

First of all, it is important to make the distinction between the respective contribution of the cardiac endothelial cells in the myocardial capillaries and at the endocardium (purpose of the present review) [6]. The cardiac endothelium is formed by a continuous monolayer of cells that line the cavity of the heart (endocardial endothelial cells (EECs)) and the luminal surface of the myocardial blood vessels (intramyocardial capillary endothelial cells (IMCEs)). EECs are the first of the endothelium cells to develop and originate from the cardiogenic plate by the process of vasculogenesis, whereas the IMCEs originate from the mesothelial cells of the epicardium, by angiogenesis. The luminal surface of the majority of EECs has a variety of microvilli that project into the heart cavities [7]. The large contact surface area of the endocardial endothelium with cardiac cells suggests an important sensor role for EECs [8]. Gap junctions, tight junctions, and zonula adherens are present between EECs where they play a role in rapid intercellular electrochemical coupling as well as to act as a selective barrier to limit the paracellular diffusion of molecules through the intercellular spaces, respectively [8, 9]. Golgi apparatus and endoplasmic reticulum of EECs are abundant with a great number of mitochondria surrounding the nucleus [9] suggesting that these cells are highly active metabolically. Similarities exist between EECs and IMCEs, but differences are also present between these two types of endothelial cells. An important feature of endothelium is also the presence of numerous

caveolae. Caveolae are small (70–90 nm in diameter) specialized invaginations of the plasmalemmal membrane. These organelles are present in most mammalian tissues and are particularly abundant in endothelial cells. A large number of signaling molecules that regulate endothelial cells localize to caveolae (for recent review, see [10]). Caveolae are abundantly provided in caveolin-1 (Cav-1) which constitutes the nonmuscle isoform of a coat protein of caveolae. Brutsaert has reviewed in detail this point [1]. Thus, immunostaining for Cav-1 shows that the peripheral borders of EECs are nearly completely devoid of caveolin labeling, whereas IMCEs display a very intense labeling for Cav-1 [11]. Caveolin-rich plasmalemmal microdomains are sites for the constitutive nitric oxide (NO) synthase (eNOS), and the poverty of Cav-1 in these areas suggests that eNOS activity might be associated with membrane components other than caveolae or with parts of the cytoskeleton. IMCEs do not have gap junctions; thus these cells differ in the way they communicate with other adjacent endothelial and nonendothelial cells [8]. Considering their respective cytoskeleton components, stress fibers, vimentin filaments, and microtubules are found to be different in EECs and IMCEs. IMCEs contain more actin filaments or stress fibers compared to EECs. Vimentin filaments and microtubules are closely packed and aligned parallel to the cell axis in IMCEs, whereas in EECs, these components constitute an extensive filamentous network. EECs interact with components of the circulating blood entering and leaving the pulmonary vasculature and both act as an autocrine or paracrine system that modulates cardiomyocyte function and as a barrier between the superfusing blood and the cardiomyocytes. EECs and IMCEs play a role in controlling the contractility of cardiomyocytes by releasing various factors. In the normal adult heart, cardiac endothelial cells produce NO through the activity of eNOS, endothelin 1 (ET-1) after conversion of pre-proET-1 to proET-1 and into ET-1 by the ET converting enzyme, eicosanoids as prostaglandins, transform angiotensin I (Ang I) into active angiotensin II (Ang II) and release peptide growth factors and neuregulin-1 (Figure 1).

2. eNOS and NO

The eNOS is mainly present in both EECs and IMCEs [11], but to lesser extent also in cardiomyocytes [12]. Immunostaining experiments have shown that there is a considerable nonuniformity of eNOS expression between EECs and IMCEs. eNOS is generally associated with the particulate fraction in endothelial cells, in particular with the Golgi complex and with domains of the plasma membrane, the caveolae. The more intense eNOS staining in EECs compared to IMCEs appeared to be associated with more intensely labeled and larger Golgi complexes. Heterogeneity is characteristic also for eNOS labeling of the peripheral cell borders between EECs and IMCEs. The eNOS-stained peripheral borders are distinct in EECs and not observed in IMCEs. NO production by the peripherally located eNOS in EECs may be involved in the regulation of paracellular permeability and suggests a greater eNOS activity in these cells than in IMCEs. These two cell types probably account for most of the NO measurable in the effluent of *in vivo* heart or whole cardiac preparations.

Studies in endothelial cells reveals that eNOS targeted to the plasmalemma releases greater amounts of NO compared with Golgi tethered eNOS [13] and the increased amount of NO produced greatly influenced the mechanisms implicated in NO effects (cGMP-dependent signaling versus S-nitrosylation of target proteins with high and low concentrations of NO, respectively; see below) [14]. Considering eNOS associated with caveolae, eNOS interacts with Cav-1 that ensures the proper targeting of eNOS to caveolae and maintains eNOS in an inhibited state. This inhibition can be reversed by addition of exogenous calmodulin, suggesting a reciprocal regulation of the enzyme by Cav-1 versus activating calcium-calmodulin [15, 16]. Stimulus- or agonist-induced increases in intracellular calcium promote the dissociation of Cav-1 and eNOS complex. Binding of activated calcium-calmodulin to its consensus sequence on eNOS initiates catalytic activity. The phenotype of mice deficient in Cav-1 exhibits a marked hyporesponsiveness to constrictor agonists attributable to an increase of NO release [17, 18]. It has also been shown that statins potentiate eNOS activity by decreasing Cav-1 abundance *in vitro* and *in vivo*, at least in macrovascular endothelial cells where the caveolin pool is lower and the proportion of caveolin-bound eNOS is higher [19] (see also review in [4]). However, another protein called NOSIP (for eNOS-interacting protein) has been also described to be able to target eNOS to caveolae in endothelial cells of the cardiac microvasculature in rat heart [20]. These authors suggest that NOSIP is a novel type of modulator that promotes translocation of eNOS from the plasma membrane to intracellular sites, thereby uncoupling eNOS from plasma membrane caveolae and inhibiting NO synthesis.

Experiments in cultured endothelial cells have demonstrated that eNOS expression can be modulated by many factors including shear stress, TGF-β, protein kinase C, TNF-α, oxygen, and the proliferative state. The ubiquitous 90 kDa, heat-shock protein (Hsp90), is expressed at high levels (accounting for up to 1-2% of total cellular protein content) in the cytosol even in unstressed conditions. Hsp90 functions as a chaperone for the proper folding of specific protein substrates include many signal transducing molecules (e.g., nonreceptor tyrosine kinases, transcription factors, and eNOS, among others; for a review, see [21]). Most of its regulatory action in eNOS signaling has been described in endothelial cells. Hsp90 is associated with eNOS in resting endothelial cells, and upon stimulation with vascular endothelial growth factor (VEGF), estrogen, histamine, shear stress, and statins, the association between the two proteins is increased, resulting in enhanced NO production [22]. The protein kinase Akt, the kinase involved in the phosphorylation of eNOS on the active site serine 1177, is another scaffold protein for Hsp90. Akt binds to a sequence of Hsp90 that does not overlap with that involved in the binding of eNOS. Therefore, Hsp90 is proposed as an adaptor between Akt and its substrate, eNOS, thereby promoting the phosphorylation of eNOS at the active site (for more details, see [23]).

The main physiological source of NO in normal, adult nonstressed cardiac tissue is eNOS from EECs and IMCEs.

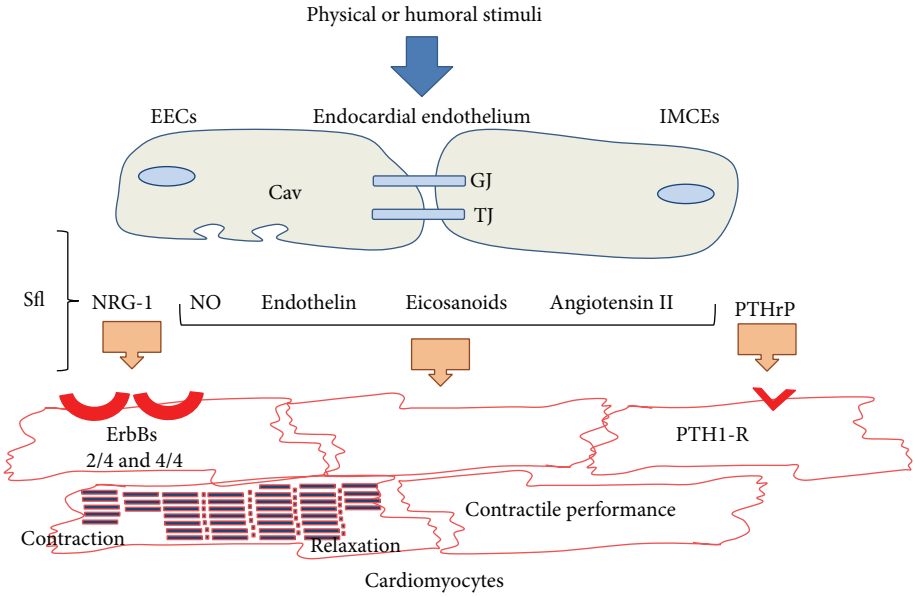

Figure 1: Paracrine communication between cardiac endothelial cells and cardiomyocytes. Endocardial endothelial cells (EECs) and intramyocardial capillary endothelial cells (IMCEs) respond to physical and humoral stimuli with the release of mediators such as nitric oxide (NO), endothelin 1, eicosanoid, and angiotensin II. These endothelial cells also release neuregulin-1 (NRG-1) acting on ErbB receptors on myocardial cardiomyocytes. IMCEs release parathyroid hormone-related peptide (PTHrP) which activates PTH1 receptor (PTH1-R) located on the cardiomyocytes. Gap junctions (GJ) allow cell-to-cell coupling for rapid intercellular communication of functional demands. Tight junctions (TJ, including zonula adherens) modulate the transendocardial endothelial-permeability through intercellular clefts. A large number of endothelial signaling molecules localize to caveolae (Cav). Sfl: subendocardial fibroelastic layer (or extracellular matrix including sympathetic nerve fascicles).

The effects of NO on myocardial contraction and relaxation have been very much studied and the signaling pathways in the heart have been reviewed in detail [4, 24, 25]. A positive inotropic response to low concentrations of NO has been reported in several studies in isolated cardiomyocytes [26, 27], whereas it induced a negative inotropic effect only at higher concentrations [28].

Most of the negative actions of high concentrations of NO on cardiac performance are due to the activation of myocardial soluble guanylate cyclase to produce cGMP and desensitization of cardiac contractile myofilaments to calcium [29]. The positive lusitropic effect of NO has also been attributed to cGMP- and PKG-mediated phosphorylations of troponin I, subsequent to myofilament calcium desensitization, relaxation hastening, and improved distensibility [30].

NO exerts its positive inotropic effects mainly via a mechanism independent of cGMP. Very low concentrations of NO activate G proteins such as Gs, stimulating adenylyl cyclase, and the L-type calcium current (I_{CaL}) from cardiac voltage-dependent L-type calcium (VDLC) channels and Gi, hence stimulating muscarinic K^+ channels [31]. NO may also bind superoxide anions to form peroxynitrite, which exerts specific effects on cardiac voltage-dependent channels and contraction [32–35]. It is indeed important to remember that NO can exert its effects directly through the S-nitrosylation of target proteins [36–40]. A recent proteomic study identified as many as 951 unique proteins that can be S-nitrosylated in the heart [41], demonstrating its potential importance in regulating cardiac function. Indeed, direct nitrosylation of the ryanodine receptor RyR2 accounts for the enhanced excitation-contraction coupling gain and positive inotropic effect of cardiomyocyte stretch [42]. A direct S-nitrosylation of the cardiac VDLC channels through a redox switch-mediated increase in I_{CaL} could also be involved [32, 33]. Although cGMP-independent activation of adenylyl cyclase at low NO levels has been suggested [26], a cGMP-dependent increase in cAMP, through cGMP-mediated inhibition of cAMP phosphodiesterase III (PDE3) and prevention of cAMP breakdown, has been reported [43]. PDE3 and PDE4 are actually considered as critical regulators of cAMP signals in cardiomyocytes [44] (for recent review on PDEs, see [45]).

3. Endothelin-1 (ET-1)

EECs and IMCEs are the major source of ET-1 in the normal heart [46], and cardiomyocytes are its primary target. Cardiac tissue displays a high density of ET_A and ET_B receptors in cardiomyocytes and endothelial cells [47, 48]. ET-1 is one of the most potent positive inotropic agents known [49, 50]. Its positive inotropic effect has been partly explained by an enhanced affinity of the contractile proteins to calcium [51], subsequent to an intracellular alkalosis resulting from activation of the sarcolemmal Na^+/H^+ exchanger [52]. Thus, ET-1 might reverse acidosis-induced negative inotropic and lusitropic effects, without increasing intracellular calcium as it occurs with most positive inotropic agents. Moreover, it has been shown that ET-1 binds directly *in vivo* to the ET_B receptors on the endothelial surface and induces the release

of endothelium-derived relaxing factors, such as NO and prostanoids [53], rather than directly promoting myocardial inotropy through ET_A receptors on the cardiomyocytes (reviewed in [3, 5]).

Furthermore, NADPH oxidase-derived reactive oxygen species (ROS) play a physiological role in the acute regulation of cardiac contractility in the intact rat heart. Results of Kubin et al. [54] reveal that ET-1-induced increase in cardiac contractility is partially dependent on enhanced ROS generation, which in turn activates the extracellular signal regulated kinase 1/2 (ERK1/2)-p90 ribosomal S6 kinase-Na^+/H^+ exchanger-1 pathway [55]. It is known that excessive levels of ROS can modulate the activity of different proteins involved in the excitation-contraction coupling, including the sarcoplasmic reticulum (SR) Ca^{2+} release channel, the SR Ca^{2+} ATPase, and the L-type Ca^{2+} channel, by modifying sulfhydryl groups of cysteine residues [56, 57]. In contrast to pathological conditions, less is known about the role of ROS in the regulation of normal cardiac function. Some data support the existence of a relationship between contraction, oxidative metabolism, and ROS production in the cardiomyocytes. Thus, increases in contraction frequency are accompanied by enhanced oxygen consumption and ROS formation in isolated cardiomyocytes [58, 59]. However, the effect of ROS on contractile function is controversial and prior studies have produced conflicting results regarding the role of ROS and ET-1 in the regulation of contractile function in isolated cardiomyocytes. Indeed, Ang II induces ET-1 release and an increase in ROS generation, which in turn triggered an increase in contractility in cat cardiomyocytes [60]. Moreover, it has been suggested that the positive inotropic effect of exogenous ET-1 is almost exclusively dependent on ROS production in this model [61]. ET-1 has been shown to increase contractility in several species such as rat, rabbit, cat, guinea pig, and human [62, 63]. However, ET-1 can elicit both positive and negative inotropic effects in murine models. Thus, ET-1 induces a negative inotropic effect in isolated mouse cardiomyocytes [64].

4. Prostaglandins

Endothelial cells, including EECs and IMCEs, synthetize and release several prostaglandins in response to a wide variety of hormonal, chemical, immunological, and physical stimuli [65, 66]. Cyclooxygenases (COX) in both constitutive (COX-l) and inducible (COX-2) isoforms are key regulators of prostaglandin synthesis. COX-l is constitutively expressed in all endothelial cells in the heart and is believed to provide cytoprotective effects. COX-2 is nearly undetectable under normal physiological conditions but is induced in endothelial cells and in macrophages during inflammation.

Brutsaert [1] has reviewed in detail the differences between EECs and IMCEs on prostanoid productions. Mebazaa et al. [67] have reported abundant production of prostacyclin (PGI_2) and prostaglandin ($PG)E_2$ from cultured EECs from right and left bovine ventricles, with EEC production of PGI_2 being 10 times higher than that of PGE_2. On the contrary, IMCEs have been reported to release more PGE_2 than PGI_2. The reasons why IMCEs release more PGE_2 than PGI_2 are still unknown. Responses to PGI_2 and PGE_2 range from increased inotropy [68] to no effect [69], to negative inotropy [70]. *In vitro* studies on isolated papillary muscle have helped to explain some of those inconsistencies [71]. With the use of activators and inhibitors of endogenous eicosanoids and of NO in different combinations, the inotropic action of eicosanoids and NO is reciprocal. Thus, stimulation of endogenous prostaglandin (mainly PGI_2) release abolishes the inotropic effect of NO, in particular on the onset of relaxation, while demonstration of a PGI_2-induced positive inotropic (contraction-prolonging) effect is dependent on inhibition of NO synthesis. Accordingly, PGI_2 and NO interact to regulate cardiac contractile performance, by their opposing effects on the onset of myocardial relaxation. NO, PGI_2, and, to a smaller extent, PGE_2 share a number of important properties and that their synthesis and release from endothelial cells are often coupled through continuous cross talk between the NOS and COX pathways [72], although their underlying subcellular mechanisms await further clarification. Their mutual actions on the target cardiomyocytes are closely linked, largely through the effects of PGI_2 and NO in establishing cAMP-to-cGMP ratios rather than absolute intracellular concentrations of cAMP or cGMP. The localization of PGI_2 synthase at the intercalated discs favors a new function of PGI_2 in the heart [73]. Since the PGI_2-receptor (IP-receptor) is also present in the same compartment, cAMP-dependent phosphorylation of connexin 43 leading to increased electrical coupling of cardiomyocytes may play a physiological role of PGI_2 in these cells.

In mouse atria, acetylcholine induces a biphasic inotropic response; that is, a transient decrease in contractile force is followed by a late increase mediated by muscarinic M2 and M3 receptors, respectively [74]. Tanaka et al. [75] show that the positive response is mediated by prostaglandin released from the endocardial endothelium. In ventricular myocardia, it has been reported that PGF_2 induces a positive inotropic response and a rise in intracellular pH (i.e., an alkalosis) [76]. In the case of the mouse atria, Tanaka et al. [77] do not detect change in pH in response to PGF_2. Further studies are needed to better understand the inotropic effect of PGF_2.

5. Angiotensin II (Ang II)

Most of the effects of Ang II on cardiac contractile performance result from locally produced rather than from circulating Ang II [78]. Ang II is synthetized through both Ang convertase enzyme- (ACE-) dependent and ACE-independent pathways, expressed in EECs and IMCEs. Several studies have investigated whether Ang II affects cardiomyocyte contractility. As an acute response to Ang II, some authors find a negative contractile response in mouse cardiomyocytes [79]. Others report a direct positive inotropic response in isolated cardiac preparations [60, 80]. In human ventricular myocardium, Ang II has either no effect or it exerts positive inotropic responses [81, 82]. The effects of Ang II share

some features of α-adrenoceptor stimulation. For example, both agonists are able to activate PLC-dependent pathways. However, in contrast to α-adrenoceptor stimulation, Ang II induces ROS formation through NADPH oxidase activation and stimulates stress-activated pathways as well [83, 84]. On the mechanistic basis, the different responses evoked by Ang II are mediated by activation of either Gαq or Gα12/13-coupled receptors. Ang II-dependent ROS formation, probably from NADPH oxidase, induces the expression of TGF-β_1 in cardiomyocytes [83, 85, 86]. Mufti et al. [87] provide strong evidence that TGF-β_1 is a key player mediating the Ang II-dependent long-term cardiodepressive effect. Ang II causes a negative contractile effect in adult rat cardiomyoctyes and this has been found to depend on p38 MAP kinase activation via ROS-independent formation [88]. Mufti et al. [87] also report that the effects of Ang II on cell shortening are also dependent on p38 MAP kinase pathway. However, as the effect in this study depends on Ang II-dependent TGF-β_1 activation, one can expect the participation of ROS formation. The exact role of p38 MAP kinase requires further studies [89]. de Giusti et al. [90] recently reviewed that acute activation of the cardiac renin-Ang II-aldosterone system induces mitochondrial ATP-dependent K^+ channel opening and subsequently enhances the production of mitochondrial ROS. These oxidant molecules, in turn, activate membrane transporters, as the Na^+/H^+ exchanger (NHE-1) and the Na^+/HCO cotransporter (NBC) *via* the stimulation of the ROS-sensitive MAPK cascade. The stimulation of such effectors leads to an increase in cardiac contractility. The mechanism of how the activation of NHE-1 or NBC regulates cardiac contractility involves the increase in intracellular Na^+ concentration [91]. Indeed, the activation of these transporters leads to a subsequent increase in intracellular Ca^{2+} concentration due to the activation of the reverse mode of the Na^+/Ca^{2+} exchanger [92–95].

6. Peptide Growth Factors

Only preliminary data are presently available about a possible role for peptide growth factors in the performance of the adult heart. IMCEs express and release parathyroid hormone-related peptide (PTHrP) [96, 97]. PTHrP exerts a positive inotropic, chronotropic, and lusitropic effects in adult ventricular cardiomyocytes and PTHrP released during an ischemia improves the inotropy of the postischemic heart [98]. PTHrP via PTH1 receptor (PTH1-R) directly improves cardiac function and myocardial perfusion through protein kinase A/protein kinase C-dependent activation of adenylate cyclase [99]. Evidences have been provided that PTHrP may play a role regarding cardiac dysfunction during situation with NO deficiency such as menopause. Indeed, Schreckenberg et al. [100] report that chronic NO deficit is associated with a loss of the inotropic and chronotropic effect of this hormone resulting from downregulation of PTH1-R via TGFβ_1-dependent pathway in left ventricular cardiomyocytes.

7. Neuregulin-1 (NRG-1)

Neuregulin-1 (NRG-1), a growth factor released from cardiac endothelial cells, has been shown to be essential for the normal function of the adult heart (Parodi and Kuhn [101]). NRG-1 mediates its actions through activation of the extracellular domain of the tyrosine kinase receptors, ErbB. In the adult heart, NRG-1, ErB2, and ErB4 are found in cardiomyocytes. Binding of NRG-1 to ErB2 and/or ErB4 induces the formation of homo- and heterodimers on cardiomyocytes [102]. Although NRG-1 does not bind directly to ErB2, it is the favored coreceptor for heterodimerization [103]. Thus, in adult cardiomyocytes, NRG-1 signaling occurs through ErbB2/ErbB4 heterodimers and/or ErbB4/ErbB4 homodimers. The first evidence for a role of NRG-1/ErbB signaling in adult heart function comes from clinical studies in patients with metastatic mammary carcinoma, undergoing combination therapy. Trastuzumab (Herceptin), a monoclonal antibody against ErbB2 combined with an anthracycline, elicited dilated cardiomyopathy and heart failure [104]. Using targeted gene inactivation in the mouse, different studies support the hypothesis that NRG-1 plays an essential roles during development of the heart and peripheral nervous system and demonstrates that the main NRG-1 receptors *in vivo* correspond to ErbB2/ErbB4 or ErbB2/ErbB3 heteromers, respectively (for reviews, see [105, 106]). The receptors ErbB2 and ErbB4 remain expressed in adult cardiomyocytes. They are localized to the T-tubule system and intercalated discs in the vicinity of components of the excitation-contraction machinery [107–109]. Thus, both the α- and β-isoforms of NRG-1 induce a negative inotropic response and activate NOS in isolated adult rabbit papillary muscles [110]. These authors also show in neonatal rat cardiomyocytes that NRG-1 can activate Akt leading to the phosphorylation of eNOS [111]. Thus, the NRG-1/ErbB signaling pathway may have a modulatory role and could be activated in conditions of enhanced cardiac inotropism, such as in myocardial hypertrophy or during β-adrenergic overdrive. On the other hand, dynamic regulation of NRG-1 expression in EECs and IMCEs occurs *in vitro* in response to other endothelial factors. Thus, mechanical stretch or stimulation with ET-1 leads to upregulation of NRG-1, while prolonged treatment with Ang II results in downregulation of its expression [112].

8. Pathophysiological Role of Cardiac Endothelial Cells

As reviewed above, EECs and IMCEs are modulators of ventricular cardiomyocyte contractile function. Thus, damage and/or dysfunction of cardiac endothelium could have a serious impact on the development of cardiac diseases. A deficient production of NO by eNOS contributes to diastolic ventricular dysfunction and abnormalities in Ca^{2+} homeostasis. Alterations in NO generation or disruption in its targeting have been shown in various pathological conditions like atherosclerotic vascular disease, congestive heart failure (CHF), and essential hypertension (reviewed in

[2, 113–115]). Indeed, eNOS can be a major ROS generator during pathologic stress. Dysfunction of NO pathway via eNOS participates in cardiac arrhythmia. Indeed, eNOS deficient mice display a slower heart rate and increase in the transient inward current and tachycardia [116]. Although cardiomyocytes isolated from heart of eNOS deficient mice have normal resting action potential (AP) duration and VDLC current under basal condition, their response to isoproterenol is altered. Application of isoproterenol induces longer AP duration, with increases in early and delayed after depolarization, and therefore induces cardiac dysfunction [117]. It is generally known that alteration of cardiac contraction is one of the main causes of cardiac remodeling leading cardiac hypertrophy. Evidences have been provided on the role of eNOS in these pathologies. Indeed, chronic eNOS deletion induces concentric hypertrophy and worsens remodeling after pressure overload [118, 119]. Other authors using abdominal aortic banding found increased hypertrophy but less chamber dilation in eNOS deficient mice [120, 121]. The therapeutic approaches used for improving NOS function in cardiovascular diseases have used organic nitrates to increase circulating NO level *via* denitration [122–124]. Enhancement of NOS pathway using various compounds has been recently described as therapeutic approaches for treating cardiac diseases and these include arginase inhibitors, NOS activators, NOS transcription enhancers, NOS recouplers, soluble guanylate cyclase activators, PDE5 inhibitors, and antioxidants (reviewed in [115]). ET-1 may also be involved in the modulation of contractile performance in pathological states. Enhancement of ET-1 production has been detected in various cardiovascular stress including acute myocardial infarction, ischaemia, CHF, cardiogenic shock, and oxidative stress (for reviews, see [2, 3, 5]). Thus, targeting ET-1 system may be therapeutic benefit for cardiovascular diseases. Indeed, bosentan and ambrisentan, ET_A-ET_B receptor, and selective ET_A receptor antagonists, respectively, have been shown to be successful in treating right ventricular hypertrophy and right heart failure subsequent to pulmonary arterial hypertension [125, 126]. However, ET-1 receptor antagonists have not yet proven to be efficient for the treatment of CHF [5, 125].

9. Conclusions

The modes of action and the regulatory paracrine role of main mediators delivered by cardiac endothelial cells upon cardiac contractility identified in cardiomyocytes are exceedingly complex and are not fully described. Furthermore, as pointed out by and fully referenced in Brutsaert [1] "*a still higher scale of complexity in the in vivo intact heart may ensue from their interaction with other important cardiomodulatory pathways, such as the β-adrenergic or cholinergic pathways in the heart, atrial and brain natriuretic peptide activity, and circulating thyroid and aldosterone hormones.*" Moreover, differences in the contribution of transporters and pumps to Ca^{2+} homeostasis between rodents and humans should be taken into account. Thus, the challenge to translate experimental evidences obtained in single cardiomyocytes to the whole heart and into useful novel therapeutic strategies is still rather complicated. Careful evaluation of new-targeted therapeutic approaches is required not to alter important physiological signaling pathways. A relevant example illustrating such requirement is the heuristic shift that occurred in recent years, showing that ROS can act as intracellular signaling molecules playing important nonpathological roles in different physiological mechanisms (present review; see also [127]). Nevertheless, the present review summarizes some insights regarding the control of cardiac contractility by endothelial cells mediators. This information may help future works to fight against cardiac diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] D. L. Brutsaert, "Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity," *Physiological Reviews*, vol. 83, no. 1, pp. 59–115, 2003.

[2] L. Kuruvilla and C. C. Kartha, "Molecular mechanisms in endothelial regulation of cardiac function," *Molecular and Cellular Biochemistry*, vol. 253, no. 1-2, pp. 113–123, 2003.

[3] A. Mohácsi, J. Magyar, T. Bányász, and P. P. Nánási, "Effects of endothelins on cardiac and vascular cells: new therapeutic target for the future," *Current Vascular Pharmacology*, vol. 2, pp. 53–63, 2004.

[4] J.-L. Balligand, O. Feron, and C. Dessy, "eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues," *Physiological Reviews*, vol. 89, no. 2, pp. 481–534, 2009.

[5] F. M. Drawnel, C. R. Archer, and H. L. Roderick, "The role of the paracrine/autocrine mediator endothelin-1 in regulation of cardiac contractility and growth," *British Journal of Pharmacology*, vol. 168, pp. 296–317, 2013.

[6] L. J. Andries and D. L. Brutsaert, "Differences in structure between endocardial and vascular endothelium," *Journal of Cardiovascular Pharmacology*, vol. 17, no. 3, pp. S243–S246, 1991.

[7] S. G. de Hert, T. C. Gillebert, L. J. Andries, and D. L. Brutsaert, "Role of the endocardial endothelium in the regulation of myocardial function: physiologic and pathophysiologic implications," *Anesthesiology*, vol. 79, no. 6, pp. 1354–1366, 1993.

[8] D. L. Brutsaert, P. Fransen, L. J. Andries, G. W. de Keulenaer, and S. U. Sys, "Cardiac endothelium and myocardial function," *Cardiovascular Research*, vol. 38, no. 2, pp. 281–290, 1998.

[9] D. L. Brutsaert, G. W. de Keulenaer, P. Fransen et al., "The cardiac endothelium: functional morphology, development, and physiology," *Progress in Cardiovascular Diseases*, vol. 39, no. 3, pp. 239–262, 1996.

[10] G. Sowa, "Caveolae, caveolins, cavins, and endothelial cell function: new insights," *Frontiers in Physiology*, vol. 2, pp. 1–13, 2012.

[11] L. J. Andries, D. L. Brutsaert, and S. U. Sys, "Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium," *Circulation Research*, vol. 82, no. 2, pp. 195–203, 1998.

[12] J.-L. Balligand, L. Kobzik, X. Han et al., "Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial (type III) nitric oxide synthase in cardiac myocytes," *The Journal of Biological Chemistry*, vol. 270, no. 24, pp. 14582–14586, 1995.

[13] Q. Zhang, J. E. Church, D. Jagnandan, J. D. Catravas, W. C. Sessa, and D. Fulton, "Functional relevance of Golgi- and plasma membrane-localized endothelial NO synthase in reconstituted endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 5, pp. 1015–1021, 2006.

[14] J. Qian, Q. Zhang, J. E. Church, D. W. Stepp, R. D. Rudic, and D. J. R. Fulton, "Role of local production of endothelium-derived nitric oxide on cGMP signaling and S-nitrosylation," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 298, no. 1, pp. H112–H118, 2010.

[15] J. B. Michel, O. Feron, K. Sase, P. Prabhakar, and T. Michel, "Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase," *The Journal of Biological Chemistry*, vol. 272, no. 41, pp. 25907–25912, 1997.

[16] V. Rizzo, D. P. McIntosh, P. Oh, and J. E. Schnitzer, "In situ flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association," *The Journal of Biological Chemistry*, vol. 273, no. 52, pp. 34724–34729, 1998.

[17] M. Drab, P. Verkade, M. Elger et al., "Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice," *Science*, vol. 293, no. 5539, pp. 2449–2452, 2001.

[18] B. Razani, J. A. Engelman, X. B. Wang et al., "Caveolin-1 Null mice are viable but show evidence of hyperproliferative and vascular abnormalities," *The Journal of Biological Chemistry*, vol. 276, no. 41, pp. 38121–38138, 2001.

[19] O. Feron, C. Dessy, J.-P. Desager, and J.-L. Balligand, "Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease caveolin abundance," *Circulation*, vol. 103, no. 1, pp. 113–118, 2001.

[20] J. Dedio, P. König, P. Wohlfart, C. Schroeder, W. Kummer, and W. Müller-Esterl, "NOSIP, a novel modulator of endothelial nitric oxide synthase activity," *The FASEB Journal*, vol. 15, no. 1, pp. 79–89, 2001.

[21] K. Richter and J. Buchner, "Hsp90: chaperoning signal transduction," *Journal of Cellular Physiology*, vol. 188, no. 3, pp. 281–290, 2001.

[22] G. García-Cardeña, R. Fan, V. Shah et al., "Dynamic activation of endothelial nitric oxide synthase by Hsp90," *Nature*, vol. 392, no. 6678, pp. 821–824, 1998.

[23] J.-L. Balligand, "Heat shock protein 90 in endothelial nitric oxide synthase signaling: following the lead(er)?" *Circulation Research*, vol. 90, no. 8, pp. 838–841, 2002.

[24] P. B. Massion and J.-L. Balligand, "Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice," *Journal of Physiology*, vol. 546, no. 1, pp. 63–76, 2003.

[25] R. Fischmeister, L. Castro, A. Abi-Gerges, F. Rochais, and G. Vandecasteele, "Species- and tissue-dependent effects of NO and cyclic GMP on cardiac ion channels," *Comparative Biochemistry and Physiology—A Molecular and Integrative Physiology*, vol. 142, no. 2, pp. 136–143, 2005.

[26] M. G. Vila-Petroff, A. Younes, J. Egan, E. G. Lakatta, and S. J. Sollott, "Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes," *Circulation Research*, vol. 84, no. 9, pp. 1020–1031, 1999.

[27] J. W. Wegener, A. Gödecke, J. Schrader, and H. Nawrath, "Effects of nitric oxide donors on cardiac contractility in wild-type and myoglobin-deficient mice," *British Journal of Pharmacology*, vol. 136, no. 3, pp. 415–420, 2002.

[28] A. J. B. Brady, J. B. Warren, P. A. Poole-Wilson, T. J. Williams, and S. E. Harding, "Nitric oxide attenuates cardiac myocyte contraction," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 265, no. 1, pp. H176–H182, 1993.

[29] A. J. Hobbs, "Soluble guanylate cyclase: the forgotten sibling," *Trends in Pharmacological Sciences*, vol. 18, no. 12, pp. 484–491, 1997.

[30] J. Layland, J.-M. Li, and A. M. Shah, "Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes," *Journal of Physiology*, vol. 540, no. 2, pp. 457–467, 2002.

[31] N. Abi-Gerges, G. Szabo, A. S. Otero, R. Fischmeister, and P.-F. Méry, "NO donors potentiate the β-adrenergic stimulation of $I_{Ca,L}$ and the muscarinic activation of $I_{K,ACh}$ in rat cardiac myocytes," *Journal of Physiology*, vol. 540, no. 2, pp. 411–424, 2002.

[32] D. L. Campbell, J. S. Stamler, and H. C. Strauss, "Redox modulation of L-type calcium channels in ferret ventricular myocytes: dual mechanism regulation by nitric oxide and S-nitrosothiols," *Journal of General Physiology*, vol. 108, no. 4, pp. 277–293, 1996.

[33] D. Malan, R. C. Levi, G. Alloatti, A. Marcantoni, I. Bedendi, and M. P. Gallo, "Cyclic AMP and cyclic GMP independent stimulation of ventricular calcium current by peroxynitrite donors in guinea pig myocytes," *Journal of Cellular Physiology*, vol. 197, no. 2, pp. 284–296, 2003.

[34] J.-M. Chesnais, R. Fischmeister, and P.-F. Méry, "Peroxynitrite is a positive inotropic agent in atrial and ventricular fibres of the frog heart," *Journal of Physiology*, vol. 521, no. 2, pp. 375–388, 1999.

[35] R. S. Ronson, M. Nakamura, and J. Vinten-Johansen, "The cardiovascular effects and implications of peroxynitrite," *Cardiovascular Research*, vol. 44, no. 1, pp. 47–59, 1999.

[36] J. S. Stamler, S. Lamas, and F. C. Fang, "Nitrosylation: the prototypic redox-based signaling mechanism," *Cell*, vol. 106, no. 6, pp. 675–683, 2001.

[37] D. T. Hess, A. Matsumoto, S.-O. Kim, H. E. Marshall, and J. S. Stamler, "Protein S-nitrosylation: purview and parameters," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 2, pp. 150–166, 2005.

[38] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.

[39] M. C. Martínez and R. Andriantsitohaina, "Reactive nitrogen species: molecular mechanisms and potential significance in health and disease," *Antioxidants & Redox Signaling*, vol. 11, no. 3, pp. 669–702, 2009.

[40] B. Lima, M. T. Forrester, D. T. Hess, and J. S. Stamler, "S-nitrosylation in cardiovascular signaling," *Circulation Research*, vol. 106, no. 4, pp. 633–646, 2010.

[41] M. J. Kohr, A. M. Aponte, J. Sun et al., "Characterization of potential S-nitrosylation sites in the myocardium," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 4, pp. H1327–H1335, 2011.

[42] M. G. V. Petroff, S. H. Kim, S. Pepe et al., "Endogenous nitric oxide mechanisms mediate the stretch dependence of Ca^{2+} release in cardiomyocytes," *Nature Cell Biology*, vol. 3, no. 10, pp. 867–873, 2001.

[43] P.-F. Mery, C. Pavoine, L. Belhassen, F. Pecker, and R. Fischmeister, "Nitric oxide regulates cardiac Ca^{2+} current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation," *The Journal of Biological Chemistry*, vol. 268, no. 35, pp. 26286–26295, 1993.

[44] F. Rochais, G. Vandecasteele, F. Lefebvre et al., "Negative feedback exerted by cAMP-dependent protein kinase and cAMP phosphodiesterase on subsarcolemmal cAMP signals in intact cardiac myocytes: an *in vivo* study using adenovirus-mediated expression of CNG channels," *The Journal of Biological Chemistry*, vol. 279, no. 50, pp. 52095–52105, 2004.

[45] T. Keravis and C. Lugnier, "Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments," *British Journal of Pharmacology*, vol. 165, no. 5, pp. 1288–1305, 2012.

[46] R. M. Kedzierski and M. Yanagisawa, "Endothelin system: the double-edged sword in health and disease," *Annual Review of Pharmacology and Toxicology*, vol. 41, pp. 851–876, 2001.

[47] P. Molenaar, G. O'Reilly, A. Sharkey et al., "Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium," *Circulation Research*, vol. 72, no. 3, pp. 526–538, 1993.

[48] D. Jacques, S. Sader, S. Choufani, P. D'Orleans-Juste, and D. Charest, "Endothelin-1 regulates cytosolic and nuclear Ca^{2+} in human endocardial endothelium," *Journal of Cardiovascular Pharmacology*, vol. 36, no. 5, pp. S397–S400, 2000.

[49] K. Li, D. J. Stewart, and J.-L. Rouleau, "Myocardial contractile actions of endothelin-1 in rat and rabbit papillary muscles: role of endocardial endothelium," *Circulation Research*, vol. 69, no. 2, pp. 301–312, 1991.

[50] A. Mebazaa, E. Mayoux, K. Maeda et al., "Paracrine effects of endocardial endothelial cells on myocyte contraction mediated via endothelin," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 265, no. 5, pp. H1841–H1846, 1993.

[51] J. Wang, G. Paik, and J. P. Morgan, "Endothelin 1 enhances myofilament Ca^{2+} responsiveness in aequorin-loaded ferret myocardium," *Circulation Research*, vol. 69, no. 3, pp. 582–589, 1991.

[52] B. K. Kramer, T. W. Smith, and R. A. Kelly, "Endothelin and increased contractility in adult rat ventricular myocytes. Role of intracellular alkalosis induced by activation of the protein kinase C-dependent Na^+-H^+ exchanger," *Circulation Research*, vol. 68, no. 1, pp. 269–279, 1991.

[53] T. F. Luscher, C. M. Boulanger, Z. Yang, G. Noll, and Y. Dohi, "Interactions between endothelium-derived relaxing and contracting factors in health and cardiovascular disease," *Circulation*, vol. 87, no. 5, pp. V36–V44, 1993.

[54] A.-M. Kubin, R. Skoumal, P. Tavi et al., "Role of reactive oxygen species in the regulation of cardiac contractility," *Journal of Molecular and Cellular Cardiology*, vol. 50, no. 5, pp. 884–893, 2011.

[55] I. Szokodi, R. Kerkelä, A.-M. Kubin et al., "Functionally opposing roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the regulation of cardiac contractility," *Circulation*, vol. 118, no. 16, pp. 1651–1658, 2008.

[56] A. V. Zima and L. A. Blatter, "Redox regulation of cardiac calcium channels and transporters," *Cardiovascular Research*, vol. 71, no. 2, pp. 310–321, 2006.

[57] C. X. C. Santos, N. Anilkumar, M. Zhang, A. C. Brewer, and A. M. Shah, "Redox signaling in cardiac myocytes," *Free Radical Biology and Medicine*, vol. 50, no. 7, pp. 777–793, 2011.

[58] F. R. Heinzel, Y. Luo, G. Dodoni et al., "Formation of reactive oxygen species at increased contraction frequency in rat cardiomyocytes," *Cardiovascular Research*, vol. 71, no. 2, pp. 374–382, 2006.

[59] S.-I. Saitoh, C. Zhang, J. D. Tune et al., "Hydrogen peroxide: a feed-forward dilator that couples myocardial metabolism to coronary blood flow," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 12, pp. 2614–2621, 2006.

[60] H. E. Cingolani, M. C. Villa-Abrille, M. Cornelli et al., "The positive inotropic effect of angiotensin II: role of endothelin-1 and reactive oxygen species," *Hypertension*, vol. 47, no. 4, pp. 727–734, 2006.

[61] V. C. de Giusti, M. V. Correa, M. C. Villa-Abrille et al., "The positive inotropic effect of endothelin-1 is mediated by mitochondrial reactive oxygen species," *Life Sciences*, vol. 83, no. 7-8, pp. 264–271, 2008.

[62] M. Endoh, "Signal transduction and Ca^{2+} signaling in intact myocardium," *Journal of Pharmacological Sciences*, vol. 100, no. 5, pp. 525–537, 2006.

[63] F. Brunner, C. Brás-Silva, A. S. Cerdeira, and A. F. Leite-Moreira, "Cardiovascular endothelins: essential regulators of cardiovascular homeostasis," *Pharmacology and Therapeutics*, vol. 111, no. 2, pp. 508–531, 2006.

[64] K. Nishimaru, T. Arimoto, Y. Takeishi, I. Kubota, K. Ishii, and M. Endoh, "Overexpression of diacylglycerol kinase ζ inhibits endothelin-1-induced decreases in Ca^{2+} transients and cell shortening in mouse ventricular myocytes," *Journal of Molecular and Cellular Cardiology*, vol. 44, no. 3, pp. 520–526, 2008.

[65] T. D. Carter and J. D. Pearson, "Regulation of prostacyclin synthesis in endothelial cells," *NIPS*, vol. 7, pp. 64–69, 1992.

[66] M. E. Gerritsen, "Physiological and pathophysiological roles of eicosanoids in the microcirculation," *Cardiovascular Research*, vol. 32, no. 4, pp. 720–732, 1996.

[67] A. Mebazaa, R. Wetzel, M. Cherian, and M. Abraham, "Comparison between endocardial and great vessel endothelial cells: morphology, growth, and prostaglandin release," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 268, no. 1, pp. H250–H259, 1995.

[68] D. S. Damron and B. A. Summers, "Arachidonic acid enhances contraction and intracellular Ca^{2+} transients in individual rat ventricular myocytes," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 272, no. 1, pp. H350–H359, 1997.

[69] M. M. Couttenye, N. M. de Clerck, A. G. Herman, and D. L. Brutsaert, "Effects of prostacyclin on contractile properties of isolated mammalian cardiac muscle," *Journal of Cardiovascular Pharmacology*, vol. 7, no. 5, pp. 971–976, 1985.

[70] K. Schror and T. Hohlfeld, "Inotropic actions of eicosanoids," *Basic Research in Cardiology*, vol. 87, no. 1, pp. 2–11, 1992.

[71] P. Mohan, D. L. Brutsaert, and S. U. Sys, "Myocardial performance is modulated by interaction of cardiac endothelium derived nitric oxide and prostaglandins," *Cardiovascular Research*, vol. 29, no. 5, pp. 637–640, 1995.

[72] R. J. Hendrickson, C. Cappadona, E. N. Yankah, J. V. Sitzmann, P. A. Cahill, and E. M. Redmond, "Sustained pulsatile flow regulates endothelial nitric oxide synthase and cyclooxygenase expression in co-cultured vascular endothelial and smooth muscle cells," *Journal of Molecular and Cellular Cardiology*, vol. 31, no. 3, pp. 619–629, 1999.

[73] G. Klumpp, S. Schildknecht, W. Nastainczyk, V. Ullrich, and M. Bachschmid, "Prostacyclin in the cardiovascular system: new aspects and open questions," *Pharmacological Reports*, vol. 57, pp. S120–S126, 2005.

[74] K. Nishimaru, Y. Tanaka, H. Tanaka, and K. Shigenobu, "Positive and negative inotropic effects of muscarinic receptor stimulation in mouse left atria," *Life Sciences*, vol. 66, no. 7, pp. 607–615, 2000.

[75] H. Tanaka, K. Nishimaru, M. Kobayashi, T. Matsuda, Y. Tanaka, and K. Shigenobu, "Acetylcholine-induced positive inotropy mediated by prostaglandin released from endocardial endothelium in mouse left atrium," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 363, no. 5, pp. 577–582, 2001.

[76] S. Fong Yew, K. A. Reeves, and B. Woodward, "Effects of prostaglandin F(2α) on intracellular pH, intracellular calcium, cell shortening and L-type calcium currents in rat myocytes," *Cardiovascular Research*, vol. 40, no. 3, pp. 538–545, 1998.

[77] H. Tanaka, K. Nishimaru, R. Makuta et al., "Possible involvement of prostaglandins F2α and D2 in acetylcholine-induced positive inotropy in isolated mouse left atria," *Pharmacology*, vol. 67, no. 3, pp. 157–162, 2003.

[78] V. J. Dzau, "Circulating versus local renin-angiotensin system in cardiovascular homeostasis," *Circulation*, vol. 77, no. 6, pp. 4–13, 1988.

[79] K. Sakurai, I. Norota, H. Tanaka, I. Kubota, H. Tomoike, and M. Endoh, "Negative inotropic effects of angiotensin II, endothelin-1 and phenylephrine in indo-1 loaded adult mouse ventricular myocytes," *Life Sciences*, vol. 70, no. 10, pp. 1173–1184, 2002.

[80] A. L. Scott, R. S. L. Chang, V. J. Lotti, and P. K. S. Siegl, "Cardiac angiotensin receptors: effects of selective angiotensin II receptor antagonists, DUP 753 and PD 121981, in rabbit heart," *Journal of Pharmacology and Experimental Therapeutics*, vol. 261, no. 3, pp. 931–935, 1992.

[81] C. Schomisch Moravec, M. D. Schluchter, L. Paranandi et al., "Inotropic effects of angiotensin II on human cardiac muscle in vitro," *Circulation*, vol. 82, no. 6, pp. 1973–1984, 1990.

[82] C. Holubarsch, G. Hasenfuss, S. Schmidt-Schweda et al., "Angiotensin I and II exert inotropic effects in atrial but not in ventricular human myocardium: an in vitro study under physiological experimental conditions," *Circulation*, vol. 88, no. 3, pp. 1228–1237, 1993.

[83] S. Wenzel, G. Taimor, H. M. Piper, and K. D. Schlüter, "Redox-sensitive intermediates mediate angiotensin II-induced p38 MAP kinase activation, AP-1 binding activity, and TGF-beta expression in adult ventricular cardiomyocytes," *The FASEB Journal*, vol. 15, no. 12, pp. 2291–2293, 2001.

[84] D. J. Grieve, J. A. Byrne, A. Siva et al., "Involvement of the nicotinamide adenosine dinucleotide phosphate oxidase isoform Nox2 in cardiac contractile dysfunction occurring in response to pressure overload," *Journal of the American College of Cardiology*, vol. 47, no. 4, pp. 817–826, 2006.

[85] G. Taimor, K.-D. Schlüter, and H. M. Piper, "Hypertrophy-associated gene induction after β-adrenergic stimulation in adult cardiomyocytes," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 3, pp. 503–511, 2001.

[86] D. Schröder, J. Heger, H. M. Piper, and G. Euler, "Angiotensin II stimulates apoptosis via TGF-β1 signaling in ventricular cardiomyocytes of rat," *Journal of Molecular Medicine*, vol. 84, no. 11, pp. 975–983, 2006.

[87] S. Mufti, S. Wenzel, G. Euler, H. M. Piper, and K.-D. Schlüter, "Angiotensin II-dependent loss of cardiac function: mechanisms and pharmacological targets attenuating this effect," *Journal of Cellular Physiology*, vol. 217, no. 1, pp. 242–249, 2008.

[88] J. Palomeque, L. Sapia, R. J. Hajjar, A. Mattiazzi, and M. Vila Petroff, "Angiotensin II-induced negative inotropy in rat ventricular myocytes: role of reactive oxygen species and p38 MAPK," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 290, no. 1, pp. H96–H106, 2006.

[89] A. Sabri and P. A. Lucchesi, "ANG II and cardiac myocyte contractility: p38 is not stressed out!," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 290, no. 1, pp. H72–H73, 2006.

[90] V. C. de Giusti, C. I. Caldiz, I. E. Ennis, N. G. Pérez, H. E. Cingolani, and E. A. Aiello, "Mitochondrial reactive oxygen species (ROS) as signaling molecules of intracellular pathways triggered by the cardiac renin-angiotensin II-aldosterone system (RAAS)," *Frontiers in Physiology*, vol. 4, pp. 1–9, 2013.

[91] R. D. Vaughan-Jones, F. C. Villafuerte, P. Swietach, T. Yamamoto, A. Rossini, and K. W. Spitzer, "pH-regulated Na^+ influx into the mammalian ventricular myocyte: the relative role of Na^+-H^+ exchange and Na^+-$HCO^3\_$ Co-transport," *Journal of Cardiovascular Electrophysiology*, vol. 17, no. 1, pp. S134–S140, 2006.

[92] N. G. Pérez, M. C. C. de Hurtado, and H. E. Cingolani, "Reverse mode of the Na^+-Ca^{2+} exchange after myocardial stretch: underlying mechanism of the slow force response," *Circulation Research*, vol. 88, no. 4, pp. 376–382, 2001.

[93] E. C. Rothstein, K. L. Byron, R. E. Reed, L. Fliege, and P. A. Lucchesi, "H_2O_2-induced Ca^{2+} overload in NRVM involves $ERK_{1/2}$ MAP kinases: role for an NHE-1-dependent pathway," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 283, no. 2, pp. H598–H605, 2002.

[94] A. Bril, "Ion transporters and cardiovascular diseases: pH control or modulation of intracellular calcium concentration," *Annales de Cardiologie et d'Angeiologie*, vol. 52, no. 1, pp. 41–51, 2003.

[95] P. E. Morgan, M. V. Correa, I. E. Ennis, A. A. Diez, N. G. Pérez, and H. E. Cingolani, "Silencing of sodium/hydrogen exchanger in the heart by direct injection of naked siRNA," *Journal of Applied Physiology*, vol. 111, no. 2, pp. 566–572, 2011.

[96] K.-D. Schlüter, "PTH and PTHrP: similar structures but different functions," *News in Physiological Sciences*, vol. 14, no. 6, pp. 243–248, 1999.

[97] K.-D. Schlüter, C. Katzer, K. Frischkopf, S. Wenzel, G. Taimor, and H. M. Piper, "Expression, release, and biological activity of parathyroid hormone- related peptide from coronary endothelial cells," *Circulation Research*, vol. 86, no. 9, pp. 946–951, 2000.

[98] J. Jansen, P. Gres, C. Umschlag et al., "Parathyroid hormone-related peptide improves contractile function of stunned myocardium in rats and pigs," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 284, no. 1, pp. H49–H55, 2003.

[99] K.-D. Schlüter, M. Weber, and H. M. Piper, "Effects of PTHrP (107-111) and PTHrP(7-34) on adult cardiomyocytes," *Journal of Molecular and Cellular Cardiology*, vol. 29, pp. 3057–3065, 1997.

[100] R. Schreckenberg, S. Wenzel, R. M. D. C. Rebelo, A. Röthig, R. Meyer, and K.-D. Schlüter, "Cell-specific effects of nitric oxide

deficiency on parathyroid hormone-related peptide (PTHrP) responsiveness and PTH1 receptor expression in cardiovascular cells," *Endocrinology*, vol. 150, no. 8, pp. 3735–3741, 2009.

[101] E. M. Parodi and B. Kuhn, "Signaling between microvascular endothelium and cardiomyocytes through neuregulin," *Cardiovascular Research*, 2014.

[102] T. Holbro and N. E. Hynes, "ErbB receptors: directing key signaling networks throughout life," *Annual Review of Pharmacology and Toxicology*, vol. 44, pp. 195–217, 2004.

[103] S. Burden and Y. Yarden, "Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis," *Neuron*, vol. 18, no. 6, pp. 847–855, 1997.

[104] J. W. Schneider, A. Y. Chang, and A. Garratt, "Trastuzumab cardiotoxicity: speculations regarding pathophysiology and targets for further study," *Seminars in Oncology*, vol. 29, no. 3, pp. 22–28, 2002.

[105] D. L. Falls, "Neuregulins: functions, forms, and signaling strategies," *Experimental Cell Research*, vol. 284, no. 1, pp. 14–30, 2003.

[106] A. N. Garratt, C. Özcelik, and C. Birchmeier, "ErbB2 pathways in heart and neural diseases," *Trends in Cardiovascular Medicine*, vol. 13, no. 2, pp. 80–86, 2003.

[107] S. A. Crone, Y.-Y. Zhao, L. Fan et al., "ErbB2 is essential in the prevention of dilated cardiomyopathy," *Nature Medicine*, vol. 8, no. 5, pp. 459–465, 2002.

[108] C. Özcelik, B. Erdmann, B. Pilz et al., "Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 13, pp. 8880–8885, 2002.

[109] H. Ueda, A. Oikawa, A. Nakamura, F. Terasawa, K. Kawagishi, and T. Moriizumi, "Neuregulin receptor ErbB2 localization at T-tubule in cardiac and skeletal muscle," *Journal of Histochemistry and Cytochemistry*, vol. 53, no. 1, pp. 87–91, 2005.

[110] K. Lemmens, P. Fransen, S. U. Sys, D. L. Brutsaert, and G. W. de Keulenaer, "Neuregulin-1 induces a negative inotropic effect in cardiac muscle: role of nitric oxide synthase," *Circulation*, vol. 109, no. 3, pp. 324–326, 2004.

[111] I. Fleming and R. Busse, "Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 284, no. 1, pp. R1–R12, 2003.

[112] K. Lemmens, V. F. M. Segers, M. Demolder, and G. W. de Keulenaer, "Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte cross-talk," *The Journal of Biological Chemistry*, vol. 281, no. 28, pp. 19469–19477, 2006.

[113] M. Seddon, A. M. Shah, and B. Casadei, "Cardiomyocytes as effectors of nitric oxide signalling," *Cardiovascular Research*, vol. 75, no. 2, pp. 315–326, 2007.

[114] B. Manoury, V. Montiel, and J.-L. Balligand, "Nitric oxide synthase in post-ischaemic remodelling: new pathways and mechanisms," *Cardiovascular Research*, vol. 94, no. 2, pp. 304–315, 2012.

[115] R. Carnicer, M. J. Crabtree, V. Sivakumaran, B. Casadei, and D. A. Kass, "Nitric oxide synthases in heart failure," *Antioxidants & Redox Signaling*, vol. 18, pp. 1078–1099, 2013.

[116] A. Rakhit, C. T. Maguire, H. Wakimoto et al., "*In vivo* electrophysiologic studies in endothelial nitric oxide synthase (eNOS)-deficient mice," *Journal of Cardiovascular Electrophysiology*, vol. 12, no. 11, pp. 1295–1301, 2001.

[117] H. Wang, M. J. Kohr, D. G. Wheeler, and M. T. Ziolo, "Endothelial nitric oxide synthase decreases β-adrenergic responsiveness via inhibition of the L-type Ca^{2+} current," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 294, no. 3, pp. H1473–H1480, 2008.

[118] L. A. Barouch, R. W. Harrison, M. W. Skaf et al., "Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms," *Nature*, vol. 416, no. 6878, pp. 337–339, 2002.

[119] M. Scherrer-Crosbie, R. Ullrich, K. D. Bloch et al., "Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice," *Circulation*, vol. 104, no. 11, pp. 1286–1291, 2001.

[120] H. Ruetten, S. Dimmeler, D. Gehring, C. Ihling, and A. M. Zeiher, "Concentric left ventricular remodeling in endothelial nitric oxide synthase knockout mice by chronic pressure overload," *Cardiovascular Research*, vol. 66, no. 3, pp. 444–453, 2005.

[121] E. Takimoto, H. C. Champion, M. Li et al., "Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load," *Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1221–1231, 2005.

[122] K. E. Torfgard and J. Ahlner, "Mechanisms of action of nitrates," *Cardiovascular Drugs and Therapy*, vol. 8, no. 5, pp. 701–717, 1994.

[123] A. Kollau, M. Beretta, M. Russwurm et al., "Mitochondrial nitrite reduction coupled to soluble guanylate cyclase activation: lack of evidence for a role in the bioactivation of nitroglycerin," *Nitric Oxide—Biology and Chemistry*, vol. 20, no. 1, pp. 53–60, 2009.

[124] A. Kollau, A. Hofer, M. Russwurm et al., "Contribution of aldehyde dehydrogenase to mitochondrial bioactivation of nitroglycerin: evidence for the activation of purified soluble guanylate cyclase through direct formation of nitric oxide," *Biochemical Journal*, vol. 385, no. 3, pp. 769–777, 2005.

[125] N. S. Kirkby, P. W. F. Hadoke, A. J. Bagnall, and D. J. Webb, "The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house?" *British Journal of Pharmacology*, vol. 153, no. 6, pp. 1105–1119, 2008.

[126] M. Gomberg-Maitland, C. Dufton, R. J. Oudiz, and R. L. Benza, "Compelling evidence of long-term outcomes in pulmonary arterial hypertension? A clinical perspective," *Journal of the American College of Cardiology*, vol. 57, no. 9, pp. 1053–1061, 2011.

[127] B. D'Autréaux and M. B. Toledano, "ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis," *Nature Reviews Molecular Cell Biology*, vol. 8, pp. 813–824, 2007.

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Review Article

Endothelial Dysfunction in Experimental Models of Arterial Hypertension: Cause or Consequence?

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Hypertension is a risk factor for other cardiovascular diseases and endothelial dysfunction was found in humans as well as in various commonly employed animal experimental models of arterial hypertension. Data from the literature indicate that, in general, endothelial dysfunction would not be the cause of experimental hypertension and may rather be secondary, that is, resulting from high blood pressure (BP). The initial mechanism of endothelial dysfunction itself may be associated with a lack of endothelium-derived relaxing factors (mainly nitric oxide) and/or accentuation of various endothelium-derived constricting factors. The involvement and role of endothelium-derived factors in the development of endothelial dysfunction in individual experimental models of hypertension may vary, depending on the triggering stimulus, strain, age, and vascular bed investigated. This brief review was focused on the participation of endothelial dysfunction, individual endothelium-derived factors, and their mechanisms of action in the development of high BP in the most frequently used rodent experimental models of arterial hypertension, including nitric oxide deficient models, spontaneous (pre)hypertension, stress-induced hypertension, and selected pharmacological and diet-induced models.

1. Introduction

Cardiovascular diseases account for about one-third of premature deaths in men and one-quarter of premature deaths in women, and arterial hypertension is one of the most significant risk factors for cardiovascular diseases. Despite current knowledge and extensive clinical and experimental research, the cause of hypertension remains unknown in about 95% of all cases. There are several factors that—alone or in combination—can increase the risk of developing primary hypertension in humans. In general, these include genetic and environmental factors.

Genetic association studies have identified polymorphisms in several candidate genes (e.g., angiotensinogen, angiotensin-converting enzyme, alpha-adducin, beta-adrenergic receptors, endothelial nitric oxide synthase (NOS), cytochrome P_{450} 2C19, and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase)) and several genomic sites that may include other genes contributing to primary hypertension [1–11]. However, none of these genetic abnormalities seems to be responsible for a significant portion of hypertension in the general population. Yet the influence of genetic factors may be accentuated and disease can be triggered by interaction of several gene polymorphisms or with environmental inputs such as sedentary life style, smoking, dietary factors (high salt, sugar, fat/cholesterol and alcohol intake, and low potassium and calcium intake) and chronic stress.

It is well known that pathophysiological characteristics of essential hypertension involve, besides other factors, increased total peripheral resistance. Thus, several experimental models of hypertension have been developed in rodents to study the mechanisms of blood pressure (BP) regulation in order to better understand the cause and consequences of human arterial hypertension [12]. These experimental models allow not only to modify all potential factors—diet, surrounding environment, and genetic information (by using specific gene knock-out or transgenic models)—but also to study the influence of interaction of specific risk factors in the etiology of hypertension. Moreover,

these models are developed to allow investigation of endothelial function of various arteries, either *in vivo* or *in vitro*, including determination of mechanisms involved in loss of normal vascular function.

Endothelium, the inner layer of the blood vessels, was originally considered to be a passive barrier between blood and the vascular wall. This opinion was broken after the discovery of prostaglandin X by Bunting and coworkers in 1976 [13]. Their study showed that the arterial wall can synthetize and release a vasoactive substance that is able to relax arterial strips and to inhibit platelet aggregation. Prostaglandin (PG) X was soon identified as PGI_2 (prostacyclin) synthetized and released by the endothelial cells [14]. However, only the discovery of the obligatory role of the endothelium in relaxation of the arterial wall by the chemically unknown "endothelium-derived relaxing factor (EDRF)" by Furchgott and Zawadski in 1980 [15] and identification of EDRF as nitric oxide (NO) started the real age of "endothelial research." Soon it became clear that prostacyclin and NO are not the only vasoactive substances released by the endothelium.

Today it is known that the endothelium produces various substances collectively termed endothelium-derived relaxing factors (EDRFs) and endothelium-derived constricting factors (EDCFs), based on their function in modulation of the arterial wall. In addition to these factors there are vasorelaxing endothelium-derived hyperpolarizing factors (EDHFs), whose chemical moiety is still under discussion. This brief, though not exhausting, review is focused on main mechanisms involved in the development of endothelial dysfunction in selected models of experimental hypertension.

2. Endothelium-Derived Factors

2.1. Endothelium-Derived Relaxing Factors. NO, PGI_2, and hydrogen sulfide (H_2S) were described as EDRFs and NO is the best characterized EDRF. In addition, there is a group of EDRFs that produce relaxation due to hyperpolarization and they all go under the term EDHFs.

In mammals NO is produced by one of four nitric oxide synthase (NOS) isoenzymes (nNOS/NOS I, that is, neuronal NOS; iNOS/NOS II, that is, inducible NOS; eNOS/NOS III, that is, endothelial NOS; or mtNOS/NOS IV, that is, mitochondrial NOS). They are involved in the modulation of various pathophysiological or diseased states. Endothelial NOS is the main isoform expressed in the endothelium. It is localized in cellular plasma membranes in the caveolae and in the membrane of the Golgi body [16]. Briefly, the regulation of NO production in the endothelium depends on bioavailability of several cofactors, phosphorylation of eNOS in specific sites, co- and posttranslational lipid modifications, and it is a subject of negative feedback regulation by NO itself [17–20]. NO can diffuse from endothelial cells to vascular smooth muscle cells and guanylate cyclase (GC) has been identified as an intracellular receptor for NO (Figure 1). Its activation leads to the release of the second messenger cyclic guanosine monophosphate (cGMP) and activation of protein kinase (PK) G-dependent mechanisms resulting in reduction of intracellular calcium concentration ($[Ca^{2+}]_i$), followed by vasorelaxation. In addition, there are many other functions of NO participating in the regulation of gene transcription, mRNA translation, and protein modification of various enzymes involved in mitochondrial respiration, mitogenesis, and growth [19, 21, 22].

As mentioned above, PGI_2 was the first endothelium-derived relaxing factor discovered. PGI_2 is the main product of arachidonic acid (AA), cyclooxygenase- (COX-) mediated metabolism in vascular tissues. In normal conditions, PGI_2 produced by prostacyclin synthase acts on the prostacyclin receptor (IP). Activation of the IP receptor leads to G protein-coupled increase in the second messenger cyclic adenosine monophosphate (cAMP) and PK A activation, resulting in decreased $[Ca^{2+}]_i$ and vasorelaxation. In the vasculature, PGI_2 inhibits cell adhesion, thrombosis, inflammation, apoptosis, and proliferation. It participates in vasorelaxation and BP regulation; however, its role in these functions is rather minor as compared to NO [23, 24]. *In vitro* studies have suggested that there is a cross-talk between NO and PGI_2 production as NO was shown to activate the enzymes involved in PGI_2 synthesis and vice versa [25, 26].

Hydrogen sulfide can be produced in the endothelial cells by cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfur esterase (3MST) [27, 28]. Genetic deletion of CSE in mice markedly reduces H_2S levels in the aorta and other tissues. Mutant mice lacking CSE display diminished endothelium-dependent vasorelaxation after muscarinic cholinergic stimulation of vascular endothelial cells and pronounced hypertension. CSE is physiologically activated by calcium-calmodulin, which is a mechanism for H_2S formation in response to vascular activation [27].

2.2. Endothelium-Derived Hyperpolarizing Factors. Although many substances produced by the endothelium act as EDHFs, there is continuous discussion on the chemical moiety and molecular signaling of individual hyperpolarization-producing factors. Substances like NO, hydrogen peroxide (H_2O_2), carbon monoxide, adenosine [29], and K^+ itself [30] possess the ability to induce hyperpolarization. Furthermore, recently published studies showed that H_2S yielded significant hyperpolarization of vascular smooth muscle cells [31, 32].

In addition to these factors, several authors included epoxyeicosatrienoic acids (EETs) in EDHFs. EETs are synthesized in endothelial cells in the AA pathway, in which AA is converted by cytochrome P_{450} (CYP) epoxygenases to 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. The targets of EETs are large conductance calcium-activated K^+ channels (BK_{Ca}) in vascular smooth muscle cells, as well as small (SK_{Ca}) and intermediate (IK_{Ca}) conductance K_{Ca} channels of endothelial cells, whose activation leads to hyperpolarization [33]. Besides regulation of vascular tone, EETs participate in vascular signaling processes involved in inflammation and angiogenesis.

The importance of individual EDHFs may vary among various vascular beds and animal species. Regarding the issue of artery size, EDHF-mediated relaxation is a dominant component of acetylcholine- (ACh) induced relaxation in the

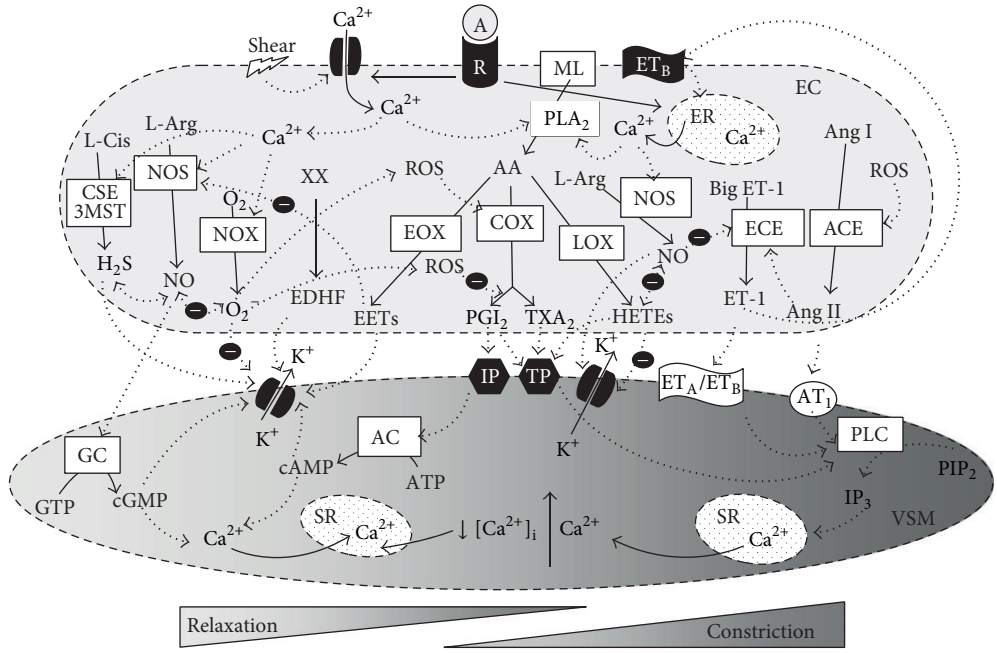

Figure 1: A brief scheme of interactions among the individual endothelium-derived factors and their mechanisms of action in the endothelial cells. Abbreviations are explained in the list of abbreviations. The activation of the appropriate receptor by its agonist as well as shear stress leads to alterations in the intracellular calcium concentration in the endothelial cells which affect the activity of all NOS, CSE, NOX, and PLA_2 resulting in the release of NO, H_2S, ROS, and AA-derived metabolites, respectively. ROS can further inhibit (marked by "−" sign) the production of PGI_2 and to elevate TXA_2 and Ang-II production. In addition, there are significant interactions among the NO, H_2S, and superoxide as well as among NO, Ang-II, ET-1, and HETEs. Individual EDRFs then affect the vascular smooth muscle cells, via modulation of the appropriate receptors or channels, resulting in the respective vascular smooth muscle cell relaxation and constriction.

small arteries, while its contribution to relaxation of the aorta is minor [23]. Differences in the mechanism of ACh-induced relaxation were observed also in mice with disruptions in the NOS genes, that is, $eNOS^{-/-}$, $n/eNOS^{-/-}$, and $n/i/eNOS^{-/-}$ mice [34]. Studies of Takaki et al. [34] revealed that EDHF-mediated relaxation and hyperpolarization in response to ACh in the small mesenteric arteries of mice were progressively reduced as the number of disrupted NOS genes increased, whereas vascular smooth muscle function was preserved, suggesting that loss of eNOS expression alone was compensated by other NOS genes. However, $EDHF/H_2O_2$-mediated responses were completely absent in $n/i/eNOS^{-/-}$ mice. Studies showed that NOS isoforms (nNOS, iNOS, and eNOS), especially eNOS, produce both NO and superoxide anions, and the latter is dismutated by superoxide dismutase (SOD) to $EDHF/H_2O_2$, which elicits hyperpolarization followed by vasodilation. In addition, the authors showed that NOS uncoupling was not involved in reactive oxygen species (ROS) production, as modulation of tetrahydrobiopterin (BH_4) synthesis had no effect on EDHF-mediated relaxation, and the BH_4/dihydrobiopterin ratio was comparable in the small mesenteric arteries and the aorta. Collectively, studies of Takaki et al. [34] provided a novel concept on the diverse roles of endothelial NOS system mainly contributing to the $EDHF/H_2O_2$ responses in microvessels while serving as NO-generating system in the large arteries.

The ratio of NO-dependent and NO-independent ACh-induced relaxation in various vascular beds is, in addition to artery size, affected by the cardiovascular genotype as well as by age. We found that NO-dependent and independent components of ACh-induced relaxation were approximately equal in the femoral artery of young (7 weeks' old) normotensive male Wistar-Kyoto (WKY) rats, while NO-dependent relaxation was dominant in age-matched spontaneously hypertensive rats (SHR). Furthermore, aging reduced NO production and NO-dependent relaxation in both normotensive and hypertensive rats [35–37].

2.3. Endothelium-Derived Constricting Factors. The endothelium can produce many constrictor factors including endothelin-1 (ET-1) and angiotensin II (Ang II), which appear to be the most powerful constrictors in the vasculature. Data in the literature suggest that ET-1 acts as mediator of Ang II-produced vasoconstriction [38–40].

However, experimental studies showed that significant effects of EDCFs in hypertensive rats were associated with COX products such as thromboxane A_2 (TXA_2), prostaglandins H_2, E_2, and $F_{2\alpha}$, isoprostanes, and monohydroxyeicosatetraenoic acids (HETEs), whose effect is associated mainly with thromboxane/prostaglandin endoperoxide receptor (TP) [41, 42]. EDCF-induced responses can be eliminated, at least partially, by COX-2 inhibitors and TP receptor blockers [43–45]. Interestingly, in conditions of lack or malfunction of the IP receptor, PGI_2 can produce contraction via the TP receptor both in young [46] and aged [47] rats.

Table 1: Classification of endothelial dysfunction proposed by Evora [55]. For more details and explanation see the original article.

Endothelial dysfunction classification
(I) Etiological classification
(A) *Primary or "genotypic"*: demonstrated for example in normotensive patients with familial antecedents of essential arterial hypertension.
(B) *Secondary or "phenotypic"*: present for example in cardiovascular diseases including arterial hypertension.
(II) Functional classification
(A) *"Vasotonic"*: implying a risk of vasospasm and thrombosis.
(B) "*Vasoplegic*": associated with a pathological release of endothelium-derived relaxing factors.
(III) Evolutionary or prognostic classification
(A) *Reversible*
(B) *Partially reversible*
(C) *Irreversible*

Furthermore, ROS such as H_2O_2, hydroxyl anion, and mainly superoxide were shown to constrict the arteries by modulation of the action of other endothelium-derived factors. ROS can be produced by mitochondria, uncoupled NOS, and various oxidases—xanthine oxidase, COX, lipoxygenases (LOX), and CYP monooxygenases. Yet NADPH oxidase was shown to serve as a primary source of so called "kindling radicals" [48]. The accentuated release of ROS is considered the main factor involved in vascular aging [37]. On the other hand, optimal ROS production is required for normal cell signaling as ROS serve as second messengers involved in activation of nuclear factor kappa B (NF-κB) and further in regulation of mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinases 1 and 2 ERK1/2, p38 mitogen-activated protein kinase (p38MAPK), c-Jun N-terminal kinase (JNK), and extracellular-signal-regulated kinase 5 (ERK5), with their respective importance in cell growth, inflammation, apoptosis, and cell differentiation [49–52].

In the vasculature, ROS possess pleiotropic effects which might be variable depending on the oxidative status of the tissue. For example, H_2O_2-induced oxidative stress increased vascular TP sensitivity and predisposed segments of the small arteries to prostanoid-induced constriction. Conversely, H_2O_2-induced vasodilation was observed in the same segments in the presence of antioxidants targeting radicals downstream of H_2O_2 [53].

Thus, correct function of the endogenous antioxidant defense systems, in association with the exogenous antioxidants, is necessary for the maintenance of balance between EDRFs and EDCFs and for normal vascular function.

3. Endothelial Dysfunction and Its Classification

Besides the balance between ROS and NO, there is complex cross-talk among the individual endothelium-derived factors with the aim to maintain appropriate endothelial function (Figure 1). Dysregulation of this cross-talk can result in alteration of normal physiological processes carried out by the endothelium, including reduction of its anticoagulant and antithrombotic properties, acceleration of vascular growth and remodeling, and impairment of endothelium-dependent vasorelaxation, that is, in endothelial dysfunction (ED). Yet neither the factors and mechanisms that modulate the balance between relaxing, anticoagulant, antithrombotic, and anti-mitotic factors on the one side and constricting, proaggregatory, and promitogenic factors on the other, nor the processes in which endothelium loses its protective functions have been fully elucidated so far. Genetic predisposition and aging [35, 54], several environmental factors such as sedentary life style, smoking, improper diet, and stress might participate in the transformation of the endothelium from a protective to a "health-threatening" organ.

In the last two decades, enormous research on ED has been conducted; however, the issue whether ED is a cause (i.e., primary) or a consequence (i.e., secondary) of high BP remains still open. In fact, the increasing, but still not exhausting, knowledge on the etiology and pathophysiology of ED suggests the need of proper classification of this disorder that could facilitate the integration of current state of the art in this field. Such classification has been proposed by Evora [55] and it is briefly described in Table 1. It includes etiological, functional, and evolutionary or prognostic aspects of ED. It should however be noted that the proposed classification has not yet been widely discussed, opposed, or accepted among scientists. As mentioned later in this review, a correct etiological classification of ED might be very difficult in some experimental models of hypertension. Moreover, ED may occur in other diseased states independently of hypertension [56].

4. Endothelial Dysfunction in Experimental Hypertension

4.1. Nitric Oxide-Deficient Models of Hypertension. One of the first diseases associated with reduced bioavailability of EDRF and altered vascular function was arterial hypertension. The importance of endogenous eNOS-derived NO production in regulation of vascular function underwent increasing investigation with implementation of NOS inhibitors. Several studies reported elevation of BP after intravenous

administration of NOS inhibitors in rabbits [57], guinea pigs [58], dogs [59], monkeys [60], and rats [61, 62]. The increase in BP was confirmed also during long-term oral treatment with NOS inhibitors [63–67]. The experimental model of "N^G-nitro-L-arginine methyl ester (L-NAME)-induced" or "NO-deficient" hypertension [68] was established to investigate not only the role of NO in vascular function and BP regulation but also in maintenance of homeostasis in the whole cardiovascular system.

Briefly, the complex mechanisms responsible for BP increase in this model of hypertension involve attenuated vascular relaxation and increased contraction in different parts of the vascular tree [69–72]. In addition, chronic NOS inhibition increases endothelium-dependent contractions of the rat aorta by inducing COX-2 expression and augmenting the production of EDCFs [73, 74] as well as by accentuation of ET-1 effect. The complex mechanism of BP increase in NO-deficient hypertension involves also accentuation of the sympathetic system tone, the renin-angiotensin system, and oxidative stress [75–78]. Furthermore, NO can modulate vascular remodeling independently of BP, which contributes to the maintenance of high BP [21, 79].

In the NO-deficient model of experimental hypertension, due to chronic nonspecific inhibition of NO production, the development of ED is associated with a gradual elevation of BP. Thus, regarding the abovementioned classification, ED in this model of hypertension might be classified as primary. Indeed, functional and morphological alterations observed in the model of L-NAME-induced hypertension [79] were similar to those observed in mice, in which three NOS isoforms were disrupted, that is, n/i/eNOS$^{-/-}$ [80]. Moreover, studies that used triple NOS disrupted n/i/eNOS$^{-/-}$ mice showed that the magnitude of hypertension in the triply NOS$^{-/-}$ mice was similar to that in mice with the eNOS gene disrupted singly (eNOS$^{-/-}$) or doubly (n/eNOS$^{-/-}$ or i/eNOS$^{-/-}$) [81]. Besides hypertension, ED due to lack of NO has been shown to be involved also in other pathological states. For example, mice with single disruption of the eNOS gene (eNOS$^{-/-}$) were insulin resistant, displayed increased triglycerides and free fatty acid levels, defective mitochondrial β-oxidation, and renal dysfunction [82–85].

The abovementioned studies suggest that hypertension is a common characteristic of the lack of eNOS-produced NO. Moreover, studies pointed out the association between eNOS-derived NO, primary ED, hypertension, and metabolic disorders involved in the cluster of metabolic syndrome as well as the development of atherosclerosis. Indeed, elevated BP and altered vascular function were observed in various genetic and diet-induced models of metabolic syndrome [86].

4.2. Models of Spontaneous Genetic Hypertension. Contrary to the NO-deficient model of hypertension, studies experimenting with the genetic model of spontaneously hypertensive rats produced inconsistent results regarding the role of NO and ED in the development of hypertension. The findings of ED in SHR depend on many factors, such as age, artery type, and methods used for determination of vascular function. A similar inconsistency was observed in NO production in SHR, in which reduced [87, 88], unchanged [89], and elevated [90–94] vascular NO synthesis and/or NOS expression were observed. Our previous reviewing of the literature showed that the enormous variability in the results concerning ED in SHR might result, on the one hand, from various methodologies used for determination of vascular function. On the other hand, regardless of methodology, aging seems to be an important factor in studying ED. ED was observed mainly in adult and aged (older than 25 weeks) and not in young (less than 6 weeks' old) SHR [95]. In our studies we observed elevated NOS activity in the aorta also in borderline hypertensive rats (BHR), which were the first filial generation of offspring of one normotensive and one hypertensive parent. In these rats, borderline hypertension (with systolic BP about 140 mmHg) was recorded in adulthood [92]. We also determined positive correlation between BP and NO-dependent component of ACh-induced relaxation in the femoral artery in adult rats [96]. We revealed that ED in the femoral artery in adult BHR and SHR males resulted rather from decreased NO-independent relaxation than from a lack of NO, a finding suggested also by other authors [47, 97]. In addition, we observed a decreased NO-independent component of ACh-induced relaxation already in young (7 weeks' old) male and female SHR [36, 98]. Interestingly, in young BHR and SHR females, ED was underlined also by a decrease of the NO-dependent component of relaxation and elevated superoxide production, which was not observed in males, suggesting sex-related differences in the mechanism of ED development.

To elucidate the causal relation between ED and high BP, we used young BHR rats whose BP is significantly elevated versus WKY as early as at the age of 7 weeks. However, despite higher BP and vascular ROS production, we did not observe ED in these rats as reduced NO-dependent relaxation was fully compensated be elevated NO-independent relaxation [98]. Thus, our findings in young BHR rats are not supportive of the idea that ED precedes hypertension in this particular model of hypertension. According to the suggested ED classification, ED in BHR (and supposedly also in SHR) seems to be rather secondary and other nonendothelial mechanisms are responsible for the induction of BP increase.

However, elevated BP itself can trigger damage of endothelial function and vascular remodeling [99]. Chronic presence of high BP *per se* was found to elicit increased arterial superoxide production by activating directly a PKC-dependent NADPH oxidase pathway, but also, in part, via activation of the local renin-angiotensin system [100].

Regarding the role of ROS in the development of hypertension, in addition to the reduction of bioavailable NO, ROS may also reduce hyperpolarization and PGI_2 synthesis [35]. Elevated ROS production was observed in the aorta of young BHR and SHR [98] as well as in adult SHR [45, 101].

Recently, the role of excessive NO production by iNOS has been suggested in the development of ED. Elevated expression of iNOS was observed already in young SHR rats but not in WKY and inhibition of iNOS prevented BP increase [87, 102]. Yet whether iNOS activation participates in ED development has to be further investigated as normal iNOS expression was detected in the small mesenteric arteries

from SHR [45]. However, in association with high NO production observed in SHR, elevated ROS may result in formation of peroxynitrite, thereby promoting nitrosative stress and ED. The interaction between NO and superoxide occurs at an extremely rapid rate of 6.7×10^9 mol/L^{-1}·s^{-1} and is about 3-times faster than the reaction rate for superoxide with SOD [103].

ROS have been implicated also in the mechanism of AA-derived EDCFs-mediated development of ED in genetic hypertension due to activation of COX [104]. ACh-induced constrictions were observed in adult SHR and BHR as well as in aged WKY [105]. As ACh led to release of PGI_2, PGH_2, as well as PGE_2 and $PGF_{2\alpha}$ acting via TP receptors in the aorta [46, 105], all these substances may be involved in EDCF-induced ED in SHR. The contribution of EDCFs to ED was determined also in other arteries (mesenteric, renal, basilar, carotid, and others), yet the nature of EDCFs might be different from that observed in the aorta [105].

Regarding the mechanism of triggering elevated EDCFs release, accentuated accumulation of calcium in the endothelial cells was found to be an initial prerequisite. As intracellular calcium is required also for eNOS [106] and NADPH oxidase activation [107], it seems plausible that defective calcium handling or signaling in the endothelium [108], resulting in abnormal calcium accumulation, might account for the acceleration of EDCFs production resulting in ED. Then even mild ED, in association with increased calcium influx and sensitivity observed in the vascular smooth muscle cells of SHR [109], can markedly facilitate contractility and initiate BP increase.

4.3. Pharmacological and Diet-Induced Models of Hypertension. In addition to the abovementioned models of hypertension, there are various pharmacological and diet-induced models. These models allow investigating the involvement of specific factors (salt, corticoids, lead, sugar, etc.) and pathways which may affect endothelial function and thus trigger the development of hypertension. Although the exact disorders in signal transduction resulting in hypertension in the individual models of hypertension may not be known, involvement of oxidative stress has been suggested in Ang II-induced hypertension [110], ET-1-induced hypertension [111], Dahl salt-induced hypertension [112], deoxycorticosterone acetate- (DOCA-) salt induced hypertension [113, 114], fructose-induced [115, 116], and lead-induced hypertension [117].

In some models reduced NO bioavailability was determined as a consequence of elevated ROS [112, 117], yet in other models of hypertension the participation of NO deficiency in ED seems to be of rather minor importance.

Numerous mechanisms implicated in ED development were observed in the model of Dahl salt-sensitive rats. Regarding EDCFs (likely PGH_2 and TXA_2), their contribution to ED was observed in carotid rings [118]. Lukaszewicz and Lombard [119] demonstrated restoration of normal vascular function in Dahl salt-sensitive rats with inhibition of the CYP4A/20-hydroxyeicosatetraenoic acid pathway, suggesting a direct role for this pathway in vascular dysfunction. Involvement of the endothelin receptor type A (ET_A)-mediated effect of ET-1 in the development of hypertension was observed in adult but not in young Dahl rats [120, 121]. Furthermore, high salt diet significantly reduced expression and activity of endothelial dimethylarginine dimethylaminohydrolase (DDAH-2), involved in asymmetric dimethylarginine (ADMA, endogenous NOS inhibitor) degradation and reduced eNOS expression, independently of BP [122]. Involvement of iNOS in modulation of endothelial function was not observed in salt-induced hypertension as W1400 (specific iNOS inhibitor) failed to modify aortic function [123]. Interestingly, in the same study NO produced by renal medullary iNOS was found to prevent excessive increases in arterial BP.

Endothelial dysfunction associated with increased aortic superoxide content, elevated NADPH oxidase activity, and decreased phosphorylated eNOS levels in aortic rings was observed also in DOCA-salt treated rats [114]. Similarly, elevated oxidative stress, yet due to activation of xanthine oxidase, was found in both the aorta and mesenteric arteries of DOCA-salt hypertensive rats in association with elevated expression of the ET_A receptor [113] and ET-1 gene [124]. Participation of elevated COX-2-derived factors that enhanced rat aortic contractility was determined in DOCA-salt treated rats by Adeagbo et al. [125]. Moreover, involvement of COX-2 mediated free radicals that impaired EDHF-mediated relaxation in the mesenteric arteries of DOCA-salt induced hypertension was also observed, while NO-dependent relaxation remained unaltered [126].

In the model of fructose-induced hypertension, the development of hypertension was associated with insulin resistance, hyperinsulinemia, and NO-dependent ED with preserved endothelium-independent vasomotion [127]. On the other hand, Lee et al. [128] did not observe reduced NO_x levels or protein expression of constitutive NOS and iNOS in the aorta of fructose-treated rats. However, their results indicate that an increased expression of vascular ET-1 may be causally related to the development of hypertension due to high fructose intake. This finding is in agreement with the observation of similar relaxing responses to ACh and sodium nitroprusside in the mesenteric arteries of fructose-treated rats in which accentuated Ang II-induced constriction was observed [129]. Finally, in contrast to salt-induced hypertension, in fructose-induced hypertension elevated ADMA levels seem to be secondary to the early development of NADPH oxidase-independent oxidative stress and elevated iNOS expression [116].

On balance, the findings in the abovementioned models of hypertension suggest that vascular NO deficiency would not be primarily involved in the hypertension development in Dahl salt-treated, DOCA-salt-treated, and fructose-treated rats while ET-1 seems to contribute significantly to ED in these models. Again, specific mechanisms can be involved in initial stages of ED in various arteries investigated.

4.4. Stress-Induced Hypertension. Stress is another important factor that has been suggested in the development of hypertension. Although the problem of stress-related hypertension

has been addressed in several studies, there are still conflicting data regarding causal relation between stress and hypertension in humans [130, 131]. In animal studies, some models of psychosocial stress were able to induce hypertension in normotensive rats [132, 133], while other models did not produce changes in BP [134–136]. In addition, animal studies revealed the importance of genetic factors in the etiology of stress-induced hypertension [136–138], thus genetically consistent rats, either normotensive or SHR, may not be always suitable for investigation of cardiovascular effects of stress. The main mechanisms involved in BP elevation in stress are associated with sympathoadrenal activation and less information is available on the role of ED in stress-induced models of hypertension.

To investigate the influence of chronic stress on vascular function, we employed the model of chronic social stress produced by crowding resulting from reduced living space. This model seems to be more relevant to the human situation [139, 140] than other stress models.

In our studies chronic crowding stress failed to increase BP in normotensive rats, either by the use of 2-week crowding in young females or of 8- or 12-week crowding in adult male WKY. Interestingly, at all time points investigated, we always found elevated vascular NO production in the aorta of stress-exposed WKY [98, 141–143]. Despite the consistency in these findings, the effect on vascular function was variable. In adult males exposed to 8-week stress, endothelium-dependent relaxation was elevated versus control in both femoral and mesenteric arteries, which was associated with improvement of NO-dependent relaxation [143, 144]. Notably, maximal ACh-induced relaxation was reduced after 12 weeks, while NO-dependent relaxation was still elevated, suggesting the development of NO-independent ED in crowded WKY. Furthermore, 2-week stress reduced NO-dependent relaxation in the femoral artery in young WKY females, but this was fully compensated by increase of the NO-independent component [98].

In contrast to normotensive rats, the same stress model led to elevation of BP in SHR and SHR-mothered BHR but not in Wistar-mothered BHR after 8-week exposure. However, stress-reduced relaxation was seen only in SHR [92]. In young females, 2-week stress led to acceleration of time-related increase of BP in BHR yet relaxation in the femoral artery was not altered despite elevated NO production [98].

Fuchs et al. [145] observed alterations in the mechanisms mediating endothelium-dependent relaxation to ACh in small mesenteric arteries isolated from adult male WKY and BHR rats after 10 days of repeated air-jet stress. In their study, decrease of NOS activity had a significantly larger inhibitory effect on ACh-induced relaxation in arteries from stressed compared with control BHR. COX-derived products contributed to ACh-induced relaxation of the small mesenteric arteries from stressed WKY rats, but not control WKY rats or BHR [145]. The same stress model led also to impairment of coronary artery relaxation in BHR [146]. In addition, the effect of air-jet on ACh-induced relaxation of the coronary artery was altered with aging in BHR. In young adult (3 months' old) BHR males exposure to stress produced an NOS-dependent increase in relaxation, while a decrease in relaxation was observed in aged (18 months' old) BHR. The impaired response to ACh observed in aged BHR was associated with superoxide anion, vasoconstrictor prostaglandins, and a loss of the component of relaxation that was NO-independent and K^+ channel mediated [147].

In 129/SV mice exposed to 28 days of stress (consisting of exposure to rat, restraint stress, and tail suspension) impaired carbachol-induced endothelium-dependent vasorelaxation, increased superoxide production, and reduced aortic eNOS levels were observed. These changes were reversed by the glucocorticoid (GCC) receptor antagonist mifepristone [148].

The involvement of GCC in disruption of NO production and/or expression was reported in studies using cultured endothelial cells. Radomski et al. showed that GCC inhibited the expression of an iNOS but not of constitutive NOS in cultured porcine vascular endothelial cells [149]. The inhibitory effect of GCC on eNOS expression and NO_x production was observed in cultured bovine coronary artery and aortic endothelial cells as well as in human umbilical vein endothelial cells [150, 151]. GCC was shown to downregulate NO production by limiting BH_4 production also in cardiac microvascular endothelial cells [152]. Moreover, GCC response elements in the eNOS promoter region were demonstrated by Liu et al. [153]. In addition to modulation of NO, GCC was shown to downregulate COX-1 expression and PGI_2 synthesis in the fetal pulmonary artery endothelial cells through activation of the glucocorticoid receptor (GR) and effects on COX-1 gene transcription [154].

Oxidative stress is another parameter that was shown to be increased by GCC. Decline in antioxidant defence by actions of corticosterone was evidenced by coordinate decreases in the activities of free-radical scavenging enzymes SOD, catalase, glutathione S-transferase, and glutathione reductase in the brain, liver, and heart of rats [155].

Surprisingly, in our studies in which elevated level of corticosterone resulted from chronic stress and not from pharmacological intervention, elevated corticosterone levels observed in adult WKY and young BHR and SHR failed to reduce NO-dependent relaxation compared to control rats [98, 143]. Our findings pointed out adapting mechanisms that play a role under chronic stress and may not be active in endothelial cell cultures and during acute stress exposure. We further considered the possible involvement of NOS isoforms different from eNOS.

NO has been previously suggested as a stress relieving molecule [156, 157] and activation of the vascular L-arginine/NO pathway may serve as an antistress system *in vivo*. Our results support the idea that elevated NO production in the vasculature can be considered one of the adaptation mechanisms protecting from sustained elevation of BP, at least in normotensive rats in allostasis. Long-term stress, however, may induce NO-independent ED that might be the initial step in the development of vascular remodelling followed by atherosclerosis and/or hypertension. Thus, in case of chronic stress-induced hypertension in genetically normotensive rats, NO-independent ED can precede hypertension and might be considered primary. Whether corticosterone is involved in the initiation of ED

in stress-induced models of hypertension, or even in spontaneous hypertension, remains to be clarified.

5. Conclusion

The conclusion that can be drawn from reviewing the literature is that ED might be both a cause and consequence of high BP, depending on the model of hypertension used, strain, age, and vascular bed. The findings in the abovementioned models of hypertension suggest that ED, due to genetic or pharmacological disruption of eNOS-derived NO, observed before sustained elevation of BP, might be considered to be primary. Such ED is causally associated with reduced NO-dependent relaxation. On the other hand, ED observed in SHR and BHR seems to be EDCF associated and NO independent, and it can be considered to be secondary ED since numerous studies confirmed the presence of elevated BP before the development of ED. Furthermore, contribution of ET-1 to ED development was predominant in diet-induced models. Yet the most prevalent cause of ED seems to be oxidative stress that has been observed in all abovementioned experimental models of hypertension. As oxidative stress may result from a broad spectrum of genetic and environmental factors as well as from high PB *per se*, it is very difficult to distinguish between primary and secondary ED in experimental models of hypertension because the processes of endothelial function damage and elevation of BP are many times occurring simultaneously.

Abbreviations

A: Agonist
AA: Arachidonic acid
AC: Adenylate Cyclase
ACE: Angiotensin-converting enzyme
ADMA: Asymmetric dimethylarginine
ACh: Acetylcholine
Ang I: Angiotensin I
Ang II: Angiotensin II
AT_1: Angiotensin II receptor 1
ATP: Adenosine-5′-triphosphate
BH_4: Tetrahydrobiopterin
BHR: Borderline hypertensive rats
BK_{Ca}: Large conductance calcium-activated K^+ channel
BP: Blood pressure
cAMP: Cyclic adenosine monophosphate
cGMP: Cyclic guanosine monophosphate
COX: Cyclooxygenase
CSE: Cystathionine γ-lyase
CYP: Cytochrome P_{450}
DDAH-2: Endothelial dimethylarginine dimethylaminohydrolase
DOCA: Deoxycorticosterone acetate
EC: Endothelial cell
ECE: Endothelin-1-converting enzyme
ED: Endothelial dysfunction
EDCF(s): Endothelium-derived constricting factor(s)
EDHF(s): Endothelium-derived hyperpolarizing factor(s)
EDRF(s): Endothelium-derived relaxing factor(s)
EET(s): Epoxyeicosatrienoic acid(s)
eNOS/NOS III: Endothelial nitric oxide synthase
EOX: Cytochrome P_{450} epoxygenases
ER: Endoplasmic reticulum
ERK1/2: Extracellular signal-regulated kinases 1 and 2
ERK5: Extracellular-signal-regulated kinase 5
ET-1: Endothelin-1
ET_A: Endothelin receptor A
ET_B: Endothelin receptor B
GC: Guanylate cyclase
GCC: Glucocorticoid
GR: Glucocorticoid receptor
GTP: Guanosine-5′-triphosphate
HETE(s): Monohydroxyeicosatetraenoic acid(s)
H_2O_2: Hydrogen peroxide
H_2S: Hydrogen sulfide
IK_{Ca}: Intermediate conductance calcium-activated K^+ channel
iNOS/NOS II: Inducible nitric oxide synthase
IP: Prostacyclin receptor
IP_3: Inositol 1,4,5-trisphosphate
JNK: c-Jun N-terminal kinase
K_{Ca}: Calcium-activated K^+ channel
L-Arg: L-arginine
L-Cis: L-cysteine
L-NAME: N^G-nitro-L-arginine methyl ester
LOX: Lipoxygenase
MAPK: Mitogen-activated protein kinase
ML: Membrane lipids
3MST: 3-Mercaptopyruvate sulfuresterase
mtNOS/NOS IV: Mitochondrial nitric oxide synthase
NADPH: Nicotinamide adenine dinucleotide phosphate
NF-κB: Nuclear factor kappa B
nNOS/NOS I: Neuronal nitric oxide synthase
NO: Nitric oxide
NOS: Nitric oxide synthase
NOX: NADPH oxidase
NO_x: Nitrate/nitrite
PG: Prostaglandin
PGI_2: Prostacyclin
PIP_2: Phosphatidylinositol 4,5-bisphosphate
PK: Protein kinase
PLC: Phospholipase C
p38MAPK: p38 mitogen-activated protein kinase
R: Receptor
ROS: Reactive oxygen species
SHR: Spontaneously hypertensive rats
SK_{Ca}: Small conductance calcium-activated K^+ channel
SOD: Superoxide dismutase
SR: Sarcoplasmic reticulum

TP: Thromboxane/prostaglandin endoperoxide receptor
TXA_2: Thromboxane A_2
VSM: Vascular smooth muscle cell
WKY: Wistar-Kyoto rats
XX: Other, not specifically mentioned substances which may produce hyperpolarization.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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References

[1] M. Baker, T. Rahman, D. Hall et al., "The C-532T polymorphism of the angiotensinogen gene is associated with pulse pressure: a possible explanation for heterogeneity in genetic association studies of AGT and hypertension," *International Journal of Epidemiology*, vol. 36, no. 6, pp. 1356–1362, 2007.

[2] W. Niu, Y. Qi, S. Hou, W. Zhou, and C. Qiu, "Correlation of angiotensin-converting enzyme 2 gene polymorphisms with stage 2 hypertension in Han Chinese," *Translational Research*, vol. 150, no. 6, pp. 374–380, 2007.

[3] P. Manunta, L. Citterio, C. Lanzani, and M. Ferrandi, "Adducin polymorphisms and the treatment of hypertension," *Pharmacogenomics*, vol. 8, no. 5, pp. 465–472, 2007.

[4] W. Mo, G. Zhang, T. Yang et al., "The genetic polymorphisms of β3-adrenergic receptor (AR) Trp64Arg and β2-AR Gln27Glu are associated with obesity in Chinese male hypertensive patients," *Clinical Chemistry and Laboratory Medicine*, vol. 45, no. 4, pp. 493–498, 2007.

[5] A. Nejatizadeh, R. Kumar, T. Stobdan et al., "Endothelial nitric oxide synthase gene haplotypes and circulating nitric oxide levels significantly associate with risk of essential hypertension," *Free Radical Biology and Medicine*, vol. 44, no. 11, pp. 1912–1918, 2008.

[6] D. Levy, A. L. DeStefano, M. G. Larson et al., "Evidence for a gene influencing blood pressure on chromosome 17: genome scan linkage results for longitudinal blood pressure phenotypes in subjects from the Framingham Heart study," *Hypertension*, vol. 36, no. 4, pp. 477–483, 2000.

[7] D. Jurkovicova, B. Sedlakova, I. Riecansky et al., "Cardiovascular diseases and molecular variants of the renin-angiotensin system components in Slovak population," *General Physiology and Biophysics*, vol. 26, no. 1, pp. 27–32, 2007.

[8] Q. He, C. Fan, M. Yu et al., "Associations of ACE gene insertion/deletion polymorphism, ACE activity, and ACE mRNA expression with hypertension in a Chinese population," *PLoS ONE*, vol. 8, Article ID e75870, 2013.

[9] Y. Ma, W. Ni, W. Zhu, Y. Xiong, and X. Deng, "Association of genetic polymorphisms of CYP 2C19 with hypertension in a Chinese Han population," *Blood Pressure*, vol. 20, no. 3, pp. 166–170, 2011.

[10] D. Petrovic, "Association of the −262C/T polymorphism in the catalase gene promoter and the C242T polymorphism of the NADPH oxidase P22phox gene with essential arterial hypertension in patients with diabetes mellitus type 2," *Clinical and Experimental Hypertension*, vol. 36, no. 1, pp. 36–39, 2014.

[11] L. N. Zhang, L. D. Ji, L. J. Fei, F. Yuan, Y. M. Zhang, and J. Xu, "Association between polymorphisms of alpha-adducin gene and essential hypertension in Chinese population," *BioMed Research International*, vol. 2013, Article ID 451094, 5 pages, 2013.

[12] J. Török, "Participation of nitric oxide in different models of experimental hypertension," *Physiological Research*, vol. 57, no. 6, pp. 813–825, 2008.

[13] S. Bunting, R. Gryglewski, S. Moncada, and J. R. Vane, "Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation," *Prostaglandins*, vol. 12, no. 6, pp. 897–913, 1976.

[14] B. B. Weksler, A. J. Marcus, and E. A. Jaffe, "Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 9, pp. 3922–3926, 1977.

[15] R. F. Furchgott and J. V. Zawadzki, "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine," *Nature*, vol. 288, no. 5789, pp. 373–376, 1980.

[16] Q. Zhang, J. E. Church, D. Jagnandan, J. D. Catravas, W. C. Sessa, and D. Fulton, "Functional relevance of Golgi- and plasma membrane-localized endothelial NO synthase in reconstituted endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 5, pp. 1015–1021, 2006.

[17] R. Rafikov, F. V. Fonseca, S. Kumar et al., "eNOS activation and NO function: structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity," *Journal of Endocrinology*, vol. 210, no. 3, pp. 271–284, 2011.

[18] J. Kopincova, A. Puzserova, and I. Bernatova, "L-NAME in the cardiovascular system—nitric oxide synthase activator?" *Pharmacological Reports*, vol. 64, pp. 511–520, 2012.

[19] U. Förstermann and W. C. Sessa, "Nitric oxide synthases: regulation and function," *European Heart Journal*, vol. 33, no. 7, pp. 829d–837d, 2012.

[20] J. Kopincová, A. Púzserová, and I. Bernátová, "Chronic low-dose L-NAME treatment effect on cardiovascular system of borderline hypertensive rats: feedback regulation?" *Neuroendocrinology Letters*, vol. 29, no. 5, pp. 784–789, 2008.

[21] I. Bernátová, O. Pecháňová, and F. Kristek, "Mechanism of structural remodelling of the rat aorta during long-term N(G)-nitro-L-arginine methyl ester treatment," *Japanese Journal of Pharmacology*, vol. 81, no. 1, pp. 99–106, 1999.

[22] U. C. Garg and A. Hassid, "Mechanisms of nitrosothio-induced antimitogenesis in aortic smooth muscle cells," *European Journal of Pharmacology*, vol. 237, no. 2-3, pp. 243–249, 1993.

[23] H. Shimokawa, H. Yasutake, K. Fujii et al., "The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation," *Journal of Cardiovascular Pharmacology*, vol. 28, no. 5, pp. 703–711, 1996.

[24] H. C. Parkington, H. A. Coleman, and M. Tare, "Prostacyclin and endothelium-dependent hyperpolarization," *Pharmacological Research*, vol. 49, no. 6, pp. 509–514, 2004.

[25] S. T. Davidge, P. N. Baker, M. K. McLaughlin, and J. M. Roberts, "Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase," *Circulation Research*, vol. 77, no. 2, pp. 274–283, 1995.

[26] K. Niwano, M. Arai, K. Tomaru, T. Uchiyama, Y. Ohyama, and M. Kurabayashi, "Transcriptional stimulation of the eNOS gene by the stable prostacyclin analogue beraprost is mediated through cAMP-responsive element in vascular endothelial cells: close link between PGI2 signal and NO pathways," *Circulation Research*, vol. 93, no. 6, pp. 523–530, 2003.

[27] G. Yang, L. Wu, B. Jiang et al., "H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine γ-lyase," *Science*, vol. 322, no. 5901, pp. 587–590, 2008.

[28] N. Skovgaard, A. Gouliaev, M. Aalling, and U. Simonsen, "The role of endogenous H_2S in cardiovascular physiology," *Current Pharmaceutical Biotechnology*, vol. 12, no. 9, pp. 1385–1393, 2011.

[29] M. Félétou and P. M. Vanhoutte, "Endothelium-dependent hyperpolarizations: past beliefs and present facts," *Annals of Medicine*, vol. 39, no. 7, pp. 495–516, 2007.

[30] G. Edwards, K. A. Dora, M. J. Gardener, C. J. Garland, and A. H. Weston, "K^+ is an endothelium-derived hyperpolarizing factor in rat arteries," *Nature*, vol. 396, no. 6708, pp. 269–272, 1998.

[31] J. Han, Z. W. Chen, and G. W. He, "Acetylcholine- and sodium hydrosulfide-induced endothelium-dependent relaxation and hyperpolarization in cerebral vessels of global cerebral ischemia-reperfusion rat," *Journal of Pharmacological Sciences*, vol. 121, pp. 318–326, 2013.

[32] G. Tang, G. Yang, B. Jiang, Y. Ju, L. Wu, and R. Wang, "H_2S is an endothelium-derived hyperpolarizing factor," *Antioxidants and Redox Signaling*, vol. 19, pp. 1634–1646, 2013.

[33] I. Fleming and R. Busse, "Endothelium-derived epoxyeicosatrienoic acids and vascular function," *Hypertension*, vol. 47, no. 4, pp. 629–633, 2006.

[34] A. Takaki, K. Morikawa, M. Tsutsui et al., "Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice," *Journal of Experimental Medicine*, vol. 205, no. 9, pp. 2053–2063, 2008.

[35] M. M. Bachschmid, S. Schildknecht, R. Matsui et al., "Vascular aging: chronic oxidative stress and impairment of redox signaling-consequences for vascular homeostasis and disease," *Annals of Medicine*, vol. 45, pp. 17–36, 2013.

[36] P. Slezak, A. Puzserova, P. Balis et al., "Genotype-related effect of crowding stress on blood pressure and vascular function in young female rats," *BioMed Research International*, vol. 2014, Article ID 413629, 11 pages, 2014.

[37] B. van der Loo, R. Labugger, J. N. Skepper et al., "Enhanced peroxynitrite formation is associated with vascular aging," *Journal of Experimental Medicine*, vol. 192, no. 12, pp. 1731–1744, 2000.

[38] E. Cediel, B. Vázquez-Cruz, J. Navarro-Cid et al., "Role of endothelin-1 and thromboxane A_2 in renal vasoconstriction induced by angiotensin II in diabetes and hypertension," *Kidney International, Supplement*, vol. 62, supplement 82, pp. S2–S7, 2002.

[39] J. Konczalla, S. Wanderer, J. Mrosek et al., "Crosstalk between the angiotensin and endothelin-system in the cerebrovasculature," *Current Neurovascular Research*, vol. 10, pp. 335–345, 2013.

[40] M. D'Amours, M. Lebel, J. H. Grose, and R. Larivière, "Renal and vascular effects of chronic nitric oxide synthase inhibition: involvement of endothelin 1 and angiotensin II," *Canadian Journal of Physiology and Pharmacology*, vol. 77, no. 1, pp. 8–16, 1999.

[41] T. F. Luscher, C. M. Boulanger, Y. Dohi, and Z. Yang, "Endothelium-derived contracting factors," *Hypertension*, vol. 19, no. 2, pp. 117–130, 1992.

[42] M. Félétou, R. A. Cohen, P. M. Vanhoutte, and T. J. Verbeuren, "TP receptors and oxidative stress. Hand in hand from endothelial dysfunction to atherosclerosis," *Advances in Pharmacology*, vol. 60, pp. 90–106, 2010.

[43] J. Li, K. A. Bian, and R. D. Bukoski, "A non-cyclo-oxygenase, non-nitric oxide relaxing factor is present in resistance arteries of normotensive but not spontaneously hypertensive rats," *American Journal of the Medical Sciences*, vol. 307, no. 1, pp. 7–14, 1994.

[44] P. M. Vanhoutte, M. Feletou, and S. Taddei, "Endothelium-dependent contractions in hypertension," *British Journal of Pharmacology*, vol. 144, no. 4, pp. 449–458, 2005.

[45] A. Virdis, R. Colucci, D. Versari et al., "Atorvastatin prevents endothelial dysfunction in mesenteric arteries from spontaneously hypertensive rats role of cyclooxygenase 2-derived contracting prostanoids," *Hypertension*, vol. 53, no. 6, pp. 1008–1016, 2009.

[46] M. Félétou, T. J. Verbeuren, and P. M. Vanhoutte, "Endothelium-dependent contractions in SHR: a tale of prostanoid TP and IP receptors," *British Journal of Pharmacology*, vol. 156, no. 4, pp. 563–574, 2009.

[47] P. Gluais, M. Lonchampt, J. D. Morrow, P. M. Vanhoutte, and M. Feletou, "Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: the Janus face of prostacyclin," *British Journal of Pharmacology*, vol. 146, no. 6, pp. 834–845, 2005.

[48] M. Majzunova, I. Dovinova, M. Barancik, and J. Y. Chan, "Redox signaling in pathophysiology of hypertension," *Journal of Biomedical Science*, vol. 20, article 69, 2013.

[49] Y. Sun and L. W. Oberley, "Redox regulation of transcriptional activators," *Free Radical Biology and Medicine*, vol. 21, no. 3, pp. 335–348, 1996.

[50] M. Karin, T. Takahashi, P. Kapahi et al., "Oxidative stress and gene expression: the AP-1 and NF-κB connections," *BioFactors*, vol. 15, no. 2–4, pp. 87–89, 2001.

[51] R. M. Touyz and E. L. Schiffrin, "Reactive oxygen species in vascular biology: implications in hypertension," *Histochemistry and Cell Biology*, vol. 122, no. 4, pp. 339–352, 2004.

[52] A. A. Alfadda and R. M. Sallam, "Reactive oxygen species in health and disease," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 936486, 14 pages, 2012.

[53] N. Puri, F. Zhang, S. R. Monu et al., "Antioxidants condition pleiotropic vascular responses to exogenous H(2)O(2): role of modulation of vascular TP receptors and the heme oxygenase system," *Antioxidants and Redox Signaling*, vol. 18, pp. 471–480, 2013.

[54] S. Liskova, M. Petrova, P. Karen, J. Kunes, and J. Zicha, "Effects of aging and hypertension on the participation of endothelium-derived constricting factor (EDCF) in norepinephrine-induced contraction of rat femoral artery," *European Journal of Pharmacology*, vol. 667, no. 1–3, pp. 265–270, 2011.

[55] P. R. B. Evora, "An open discussion about endothelial dysfunction: is it timely to propose a classification?" *International Journal of Cardiology*, vol. 73, no. 3, pp. 289–292, 2000.

[56] P. Rajendran, T. Rengarajan, J. Thangavel et al., "The vascular endothelium and human diseases," *International Journal of Biological Sciences*, vol. 9, pp. 1057–1069, 2013.

[57] D. D. Rees, R. M. J. Palmer, and S. Moncada, "Role of endothelium-derived nitric oxide in the regulation of blood pressure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 9, pp. 3375–3378, 1989.

[58] K. Aisaka, S. S. Gross, O. W. Griffith, and R. Levi, "N(G)-methylarginine, an inhibitor of endothelium-derived nitric oxide synthesis, is a potent pressor agent in the guinea pig: does nitric oxide regulate blood pressure in vivo?" *Biochemical and Biophysical Research Communications*, vol. 160, no. 2, pp. 881–886, 1989.

[59] A. Chu, D. E. Chambers, C.-C. Lin et al., "Effects of inhibition of nitric oxide formation on basal vasomotion and endothelium-dependent responses of the coronary arteries in awake dogs," *The Journal of Clinical Investigation*, vol. 87, no. 6, pp. 1964–1968, 1991.

[60] S. M. Weldon, R. J. Winquist, and J. B. Madwed, "Differential effects of L-NAME on blood pressure and heart rate responses to acetylcholine and bradykinin in cynomolgus primates," *Journal of Pharmacology and Experimental Therapeutics*, vol. 272, no. 1, pp. 126–133, 1995.

[61] M. Hecker, J. A. Mitchell, H. J. Harris, M. Katsura, C. Thiemermann, and J. R. Vane, "Endothelial cells metabolize N(G)-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine," *Biochemical and Biophysical Research Communications*, vol. 167, no. 3, pp. 1037–1043, 1990.

[62] H. M. Vargas, L. J. Ignarro, and G. Chaudhuri, "Physiological release of nitric oxide is dependent on the level of vascular tone," *European Journal of Pharmacology*, vol. 190, no. 3, pp. 393–397, 1990.

[63] M. O. Ribeiro, E. Antunes, G. de Nucci, S. M. Lovisolo, and R. Zatz, "Chronic inhibition of nitric oxide synthesis: a new model of arterial hypertension," *Hypertension*, vol. 20, no. 3, pp. 298–303, 1992.

[64] E. Delacrétaz, D. Hayoz, M. C. Osterheld, C. Y. Genton, H. R. Brunner, and B. Waeber, "Long-term nitric oxide synthase inhibition and distensibility of carotid artery in intact rats," *Hypertension*, vol. 23, no. 6, pp. 967–970, 1994.

[65] F. Kristek, M. Gerová, L. Devát, and I. Varga, "Remodelling of septal branch of coronary artery and carotid artery in L-NAME treated rats," *Physiological Research*, vol. 45, no. 4, pp. 329–333, 1996.

[66] I. Bernátová, O. Pecháňová, and F. Šimko, "Captopril prevents NO-deficient hypertension and left ventricular hypertrophy without affecting nitric oxide synthase activity in rats," *Physiological Research*, vol. 45, no. 4, pp. 311–316, 1996.

[67] J.-F. Arnal, L. Warin, and J.-B. Michel, "Determinants of aortic cyclic guanosine monophosphate in hypertension induced by chronic inhibition of nitric oxide synthase," *The Journal of Clinical Investigation*, vol. 90, no. 2, pp. 647–652, 1992.

[68] J. Dananberg, R. S. Sider, and R. J. Grekin, "Sustained hypertension induced by orally administered nitro-L-arginine," *Hypertension*, vol. 21, no. 3, pp. 359–363, 1993.

[69] R. D. Manning Jr., L. Hu, H. L. Mizelle, J.-P. Montani, and M. W. Norton, "Cardiovascular responses to long-term blockade of nitric oxide synthesis," *Hypertension*, vol. 22, no. 1, pp. 40–48, 1993.

[70] J. Török and M. Gerová, "Vascular responses after long-term inhibition of nitric oxide synthesis in newborn dogs," *Physiological Research*, vol. 45, no. 4, pp. 323–328, 1996.

[71] A. Holécyová, J. Török, I. Bernátová, and O. Pecháňová, "Restriction of nitric oxide rather than elevated blood pressure is responsible for alterations of vascular responses in nitric oxide-deficient hypertension," *Physiological Research*, vol. 45, no. 4, pp. 317–321, 1996.

[72] I. Bernátová, O. Pechánová, P. Babál, S. Kyselá, S. Stvrtina, and R. Andriantsitohaina, "Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 282, no. 3, pp. H942–H948, 2002.

[73] C. Qu, S. W. S. Leung, P. M. Vanhoutte, and R. Y. K. Man, "Chronic inhibition of nitric-oxide synthase potentiates endothelium-dependent contractions in the rat aorta by augmenting the expression of cyclooxygenase-2," *Journal of Pharmacology and Experimental Therapeutics*, vol. 334, no. 2, pp. 373–380, 2010.

[74] L. Paulis, J. Zicha, J. Kunes et al., "Regression of L-NAME-induced hypertension: the role of nitric oxide and endothelium-derived constricting factor," *Hypertension Research*, vol. 31, no. 4, pp. 793–803, 2008.

[75] O. Pecháňová, I. Bernátová, V. Pelouch, and F. Šimko, "Protein remodelling of the heart in NO-deficient hypertension: the effect of captopril," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 12, pp. 3365–3374, 1997.

[76] I. Bernátová, O. Pecháňová, and F. Šimko, "Effect of captopril in L-NAME-induced hypertension on the rat myocardium, aorta, brain and kidney," *Experimental Physiology*, vol. 84, no. 6, pp. 1095–1105, 1999.

[77] D. R. Dillenburg, C. Mostarda, I. C. Moraes-Silva et al., "Resveratrol and grape juice differentially ameliorate cardiovascular autonomic modulation in L-NAME-treated rats," *Autonomic Neuroscience*, vol. 179, no. 1-2, pp. 9–13, 2013.

[78] S. Nakmareong, U. Kukongviriyapan, P. Pakdeechote et al., "Tetrahydrocurcumin alleviates hypertension, aortic stiffening and oxidative stress in rats with nitric oxide deficiency," *Hypertension Research*, vol. 35, no. 4, pp. 418–425, 2012.

[79] P. Babál, "Chronic inhibition of NO synthesis produces myocardial fibrosis and arterial media hyperplasia," *Histology and Histopathology*, vol. 12, no. 3, pp. 623–629, 1997.

[80] M. Tsutsui, H. Shimokawa, Y. Otsuji, and N. Yanagihara, "Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases," *Pharmacology and Therapeutics*, vol. 128, no. 3, pp. 499–508, 2010.

[81] T. Monshita, M. Tsutsui, H. Shimokawa et al., "Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 30, pp. 10616–10621, 2005.

[82] P. L. Huang, "Neuronal and endothelial nitric oxide synthase gene knockout mice," *Brazilian Journal of Medical and Biological Research*, vol. 32, no. 11, pp. 1353–1359, 1999.

[83] H. Duplain, Ŕ. Burcelin, C. Sartori et al., "Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase," *Circulation*, vol. 104, no. 3, pp. 342–345, 2001.

[84] E. le Gouill, M. Jimenez, C. Binnert et al., "Endothelial nitric oxide synthase (eNOS) knockout mice have defective mitochondrial β-oxidation," *Diabetes*, vol. 56, no. 11, pp. 2690–2696, 2007.

[85] C. Yamashita, N. Tazawa, M. Ohkita, and Y. Matsumura, "Exaggerated renal pathology of partial ablation-induced chronic

renal failure in eNOS deficient mice," *Biological and Pharmaceutical Bulletin*, vol. 31, no. 5, pp. 1029–1031, 2008.

[86] A. M. Lehnen, B. Rodrigues, M. C. Irigoyen, K. de Angelis, and B. D. Schaan, "Cardiovascular changes in animal models of metabolic syndrome," *Journal of Diabetes Research*, vol. 2013, Article ID 761314, 11 pages, 2013.

[87] T. Chou, M. Yen, C. Li, and Y. Ding, "Alterations of nitric oxide synthase expression with aging and hypertension in rats," *Hypertension*, vol. 31, no. 2, pp. 643–648, 1998.

[88] Q. Yang, H. Xue, W. Wong et al., "AVE3085, an enhancer of endothelial nitric oxide synthase, restores endothelial function and reduces blood pressure in spontaneously hypertensive rats," *British Journal of Pharmacology*, vol. 163, no. 5, pp. 1078–1085, 2011.

[89] A. Kondrashov, S. Vrankova, I. Dovinova et al., "The effects of new Alibernet red wine extract on nitric oxide and reactive oxygen species production in spontaneously hypertensive rats," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 806285, 8 pages, 2012.

[90] E. Nava, G. Noll, and T. F. Luscher, "Increased activity of constitutive nitric oxide synthase in cardiac endothelium in spontaneous hypertension," *Circulation*, vol. 91, no. 9, pp. 2310–2313, 1995.

[91] N. D. Vaziri, Z. Ni, F. Oveisi, and D. L. Trnavsky-Hobbs, "Effect of antioxidant therapy on blood pressure and NO synthase expression in hypertensive rats," *Hypertension*, vol. 36, no. 6, pp. 957–964, 2000.

[92] I. Bernatova, Z. Csizmadiova, J. Kopincova, and A. Puzserova, "Vascular function and nitric oxide production in chronic social-stress-exposed rats with various family history of hypertension," *Journal of Physiology and Pharmacology*, vol. 58, no. 3, pp. 487–501, 2007.

[93] A. Púzserová, Z. Csizmadiová, and I. Bernátová, "Effect of blood pressure on L-NAME-sensitive component of vasorelaxation in adult rats," *Physiological Research*, vol. 56, supplement 2, pp. S77–S84, 2007.

[94] C. Caniffi, R. Elesgaray, M. Gironacci, C. Arranz, and M. Á. Costa, "C-type natriuretic peptide effects on cardiovascular nitric oxide system in spontaneously hypertensive rats," *Peptides*, vol. 31, no. 7, pp. 1309–1318, 2010.

[95] I. Bernatova, M. V. Conde, J. Kopincova, M. C. González, A. Puzserova, and S. M. Arribas, "Endothelial dysfunction in spontaneously hypertensive rats: focus on methodological aspects," *Journal of Hypertension*, vol. 27, pp. S27–S31, 2009.

[96] A. Puzserova, J. Kopincova, P. Slezak, P. Balis, and I. Bernatova, "Endothelial dysfunction in femoral artery of the hypertensive rats is nitric oxide independent," *Physiological Research*, vol. 62, no. 6, pp. 615–629, 2013.

[97] Y. Mori, M. Ohyanagi, S. Koida, A. Ueda, K. Ishiko, and T. Iwasaki, "Effects of endothelium-derived hyperpolariziing factor and nitric oxide on endothelial function in femoral resistance arteries of spontaneously hypertensive rats," *Hypertension Research*, vol. 29, no. 3, pp. 187–195, 2006.

[98] P. Slezak, A. Puzserova, P. Balis et al., "Genotype-related effect of crowding stress on blood pressure and vasular function in young female rats," *BioMed Research International*. In press.

[99] J. van de Voorde, B. Vanheel, and I. Leusen, "Depressed endothelium-dependent relaxation in hypertension: relation to increased blood pressure and reversibility," *Pflugers Archiv European Journal of Physiology*, vol. 411, no. 5, pp. 500–504, 1988.

[100] Z. Ungvari, A. Csiszar, P. M. Kaminski, M. S. Wolin, and A. Koller, "Chronic high pressure-induced arterial oxidative stress: involvement of protein kinase C-dependent NAD(P)H oxidase and local renin-angiotensin system," *American Journal of Pathology*, vol. 165, no. 1, pp. 219–226, 2004.

[101] L. Condezo-Hoyos, S. M. Arribas, F. Abderrahim et al., "Liver growth factor treatment reverses vascular and plasmatic oxidative stress in spontaneously hypertensive rats," *Journal of Hypertension*, vol. 30, pp. 1185–1194, 2012.

[102] C. F. Huang, C. N. Hsu, S. J. Chien, Y. J. Lin, L. T. Huang, and Y. L. Tain, "Aminoguanidine attenuates hypertension, whereas 7-nitroindazole exacerbates kidney damage in spontaneously hypertensive rats: the role of nitric oxide," *European Journal of Pharmacology*, vol. 699, pp. 233–240, 2013.

[103] L. Thomson, M. Trujillo, R. Telleri, and R. Radi, "Kinetics of cytochrome c^{2+} oxidation by peroxynitrite implications for superoxide measurements in nitric oxide-producing biological systems," *Archives of Biochemistry and Biophysics*, vol. 319, no. 2, pp. 491–497, 1995.

[104] J. Korbecki, I. Baranowska-Bosiacka, I. Gutowska, and D. Chlubek, "The effect of reactive oxygen species on the synthesis of prostanoids from arachidonic acid," *Journal of Physiology and Pharmacology*, vol. 64, pp. 409–421, 2013.

[105] R. L. Matz, M. A. de Sotomayor, C. Schott, J.-C. Stoclet, and R. Andriantsitohaina, "Vascular bed heterogeneity in age-related endothelial dysfunction with respect to NO and eicosanoids," *British Journal of Pharmacology*, vol. 131, no. 2, pp. 303–311, 2000.

[106] U. Forstermann, J. S. Pollock, H. H. H. W. Schmidt, M. Heller, and F. Murad, "Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 5, pp. 1788–1792, 1991.

[107] D. Pandey, J. Gratton, R. Rafikov, S. M. Black, and D. J. R. Fulton, "Calcium/calmodulin-dependent kinase II mediates the phosphorylation and activation of NADPH oxidase 5," *Molecular Pharmacology*, vol. 80, no. 3, pp. 407–415, 2011.

[108] S. K. Sonkusare, A. D. Bonev, J. Ledoux et al., "Elementary Ca^{2+} signals through endothelial TRPV4 channels regulate vascular function," *Science*, vol. 336, no. 6081, pp. 597–601, 2012.

[109] Z. Ungvari and A. Koller, "Endothelin and prostaglandin H_2/thromboxane A_2 enhance myogenic constriction in hypertension by increasing Ca^{2+} sensitivity of arteriolar smooth muscle," *Hypertension*, vol. 36, no. 5, pp. 856–861, 2000.

[110] H. Wakui, T. Dejima, K. Tamura et al., "Activation of angiotensin II type 1 receptor-associated protein exerts an inhibitory effect on vascular hypertrophy and oxidative stress in angiotensin II-mediated hypertension," *Cardiovascular Research*, vol. 100, pp. 511–519, 2013.

[111] A. A. Elmarakby, E. D. Loomis, J. S. Pollock, and D. M. Pollock, "NADPH oxidase inhibition attenuates oxidative stress but not hypertension produced by chronic ET-1," *Hypertension*, vol. 45, no. 2, pp. 283–287, 2005.

[112] M. Zhou, I. H. Schulman, P. J. Pagano, E. A. Jaimes, and L. Raij, "Reduced NAD(P)H oxidase in low renin hypertension: link among angiotensin II, atherogenesis, and blood pressure," *Hypertension*, vol. 47, no. 1, pp. 81–86, 2006.

[113] E. C. Viel, K. Benkirane, D. Javeshghani, R. M. Touyz, and E. L. Schiffrin, "Xanthine oxidase and mitochondria contribute to vascular superoxide anion generation in DOCA-salt hypertensive rats," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 295, no. 1, pp. H281–H288, 2008.

[114] M. Gómez-Guzmán, R. Jiménez, M. Sánchez et al., "Epicatechin lowers blood pressure, restores endothelial function, and

decreases oxidative stress and endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension," *Free Radical Biology and Medicine*, vol. 52, no. 1, pp. 70–79, 2012.

[115] N. A. Al-Awwadi, C. Araiz, A. Bornet et al., "Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 1, pp. 151–157, 2005.

[116] C. Korandji, M. Zeller, J. C. Guilland et al., "Time course of asymmetric dimethylarginine (ADMA) and oxidative stress in fructose-hypertensive rats: a model related to metabolic syndrome," *Atherosclerosis*, vol. 214, no. 2, pp. 310–315, 2011.

[117] N. D. Vaziri and D. A. Sica, "Lead-induced hypertension: role of oxidative stress," *Current Hypertension Reports*, vol. 6, no. 4, pp. 314–320, 2004.

[118] M. Zhou, Y. Nishida, Q. Chen, and H. Kosaka, "Endothelium-derived contracting factor in carotid artery of hypertensive Dahl rats," *Hypertension*, vol. 34, no. 1, pp. 39–43, 1999.

[119] K. M. Lukaszewicz and J. H. Lombard, "Role of the CYP4A/20-HETE pathway in vascular dysfunction of the Dahl salt-sensitive rat," *Clinical Science*, vol. 124, pp. 695–700, 2013.

[120] M. Barton, L. V. D'Uscio, S. Shaw, P. Meyer, P. Moreau, and T. F. Lüscher, "ET(A) receptor blockade prevents increased tissue endothelin-1, vascular hypertrophy, and endothelial dysfunction in salt-sensitive hypertension," *Hypertension*, vol. 31, no. 1, pp. 499–504, 1998.

[121] J. Zicha, Z. Dobešová, J. Kuneš, and I. Vaněčková, "Chronic endothelin A receptor blockade attenuates contribution of sympathetic nervous system to salt hypertension development in adult but not in young Dahl rats," *Acta Physiologica*, vol. 205, no. 1, pp. 124–132, 2012.

[122] Y. Cao, J. J. Mu, Y. Fang, Z. Y. Yuan, and F. Q. Liu, "Impact of high salt independent of blood pressure on PRMT/ADMA/DDAH pathway in the aorta of Dahl salt-sensitive rats," *International Journal of Molecular Sciences*, vol. 14, pp. 8062–8072, 2013.

[123] N. Tian, A. W. Gannon, R. A. Khalil, and R. D. Manning Jr., "Mechanisms of salt-sensitive hypertension: role of renal medullary inducible nitric oxide synthase," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 284, no. 2, pp. R372–R379, 2003.

[124] R. Lariviere, L. Y. Deng, R. Day, P. Sventek, G. Thibault, and E. L. Schiffrin, "Increased endothelin-1 gene expression in the endothelium of coronary arteries and endocardium in the DOCA-salt hypertensive rat," *Journal of Molecular and Cellular Cardiology*, vol. 27, no. 10, pp. 2123–2131, 1995.

[125] A. S. O. Adeagbo, X. Zhang, D. Patel et al., "Cyclo-oxygenase-2, endothelium and aortic reactivity during deoxycorticosterone acetate salt-induced hypertension," *Journal of Hypertension*, vol. 23, no. 5, pp. 1025–1036, 2005.

[126] A. S. O. Adeagbo, I. G. Joshua, C. Falkner, and P. J. Matheson, "Tempol, an antioxidant, restores endothelium-derived hyperpolarizing factor-mediated vasodilation during hypertension," *European Journal of Pharmacology*, vol. 481, no. 1, pp. 91–100, 2003.

[127] A. S. Dumont, M. E. Hyndman, R. J. Dumont et al., "Improvement of endothelial function in insulin-resistant carotid arteries treated with pravastatin," *Journal of Neurosurgery*, vol. 95, no. 3, pp. 466–471, 2001.

[128] D. H. Lee, J. Lee, D. G. Kang, Y. W. Paek, D. J. Chung, and M. Y. Chung, "Increased vascular endothelin-1 gene expression with unaltered nitric oxide synthase levels in fructose-induced hypertensive rats," *Metabolism*, vol. 50, no. 1, pp. 74–78, 2001.

[129] J. Navarro-Cid, R. Maeso, F. Perez-Vizcaino et al., "Effects of losartan on blood pressure, metabolic alterations, and vascular reactivity in the fructose-induced hypertensive rat," *Hypertension*, vol. 26, no. 6, pp. 1074–1078, 1995.

[130] M. Esler, N. Eikelis, M. Schlaich et al., "Chronic mental stress is a cause of essential hypertension: presence of biological markers of stress," *Clinical and Experimental Pharmacology and Physiology*, vol. 35, no. 4, pp. 498–502, 2008.

[131] F. Sparrenberger, S. C. Fuchs, L. B. Moreira, and F. D. Fuchs, "Stressful life events and current psychological distress are associated with self-reported hypertension but not with true hypertension: results from a cross-sectional population-based study," *BMC Public Health*, vol. 8, article 357, 2008.

[132] D. S. Fokkema, J. M. Koolhaas, J. van der Meulen, and R. Schoemaker, "Social stress induced pressure breathing and consequent blood pressure oscillation," *Life Sciences*, vol. 38, no. 6, pp. 569–575, 1986.

[133] E. Andrews, C. Jenkins, D. Seachrist, G. Dunphy, and D. Ely, "Social stress increases blood pressure and cardiovascular pathology in a normotensive rat model," *Clinical and Experimental Hypertension*, vol. 25, no. 2, pp. 85–101, 2003.

[134] S. B. Harrap, W. J. Louis, and A. E. Doyle, "Failure of psychosocial stress to induce chronic hypertension in the rat," *Journal of Hypertension*, vol. 2, no. 6, pp. 653–662, 1984.

[135] V. Lemaire and P. Mormède, "Telemetered recording of blood pressure and heart rate in different strains of rats during chronic social stress," *Physiology and Behavior*, vol. 58, no. 6, pp. 1181–1188, 1995.

[136] J. A. Mansi and G. Drolet, "Chronic stress induces sensitization in sympathoadrenal responses to stress in borderline hypertensive rats," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 272, no. 3, pp. R813–R820, 1997.

[137] B. J. Sanders and J. E. Lawler, "The borderline hypertensive rat (BHR) as a model for environmentally- induced hypertension: a review and update," *Neuroscience and Biobehavioral Reviews*, vol. 16, no. 2, pp. 207–217, 1992.

[138] I. Bernatova and Z. Csizmadiova, "Effect of chronic social stress on nitric oxide synthesis and vascular function in rats with family history of hypertension," *Life Sciences*, vol. 78, no. 15, pp. 1726–1732, 2006.

[139] I. Fleming, A. Baum, L. M. Davidson, E. Rectanus, and S. McArdle, "Chronic stress as a factor in physiologic reactivity to challenge," *Health Psychology*, vol. 6, no. 3, pp. 221–237, 1987.

[140] D. A. D'Atri, E. F. Fitzgerald, S. V. Kasl, and A. M. Ostfeld, "Crowding in prison: the relationship between changes in housing mode and blood pressure," *Psychosomatic Medicine*, vol. 43, no. 2, pp. 95–105, 1981.

[141] A. Púzserová, Z. Csizmadiová, R. Andriantsitohaina, and I. Bernátová, "Vascular effects of red wine polyphenols in chronic stress-exposed Wistar-Kyoto rats," *Physiological Research*, vol. 55, supplement 1, pp. S39–S47, 2006.

[142] I. Bernatova, A. Puzserova, and M. Dubovicky, "Sex differences in social stress-induced pressor and behavioral responses in normotensive and prehypertensive rats," *General Physiology and Biophysics*, vol. 29, no. 4, pp. 346–354, 2010.

[143] A. Puzserova, P. Slezak, P. Balis, and I. Bernatova, "Long-term social stress induces nitric oxide-independent endothelial dysfunction in normotensive rats," *Stress*, vol. 16, pp. 331–339, 2013.

[144] I. Bernátová, A. Púzserová, J. Navarová, Z. Csizmadiová, and M. Zeman, "Crowding-induced alterations in vascular system of Wistar-Kyoto rats: role of nitric oxide," *Physiological Research*, vol. 56, no. 5, pp. 667–669, 2007.

[145] L. C. Fuchs, A. M. Hoque, and N. L. Clarke, "Vascular and hemodynamic effects of behavioral stress in borderline hypertensive and Wistar-Kyoto rats," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 274, no. 2, pp. R375–R382, 1998.

[146] L. C. Fuchs, S. K. Landas, and A. K. Johnson, "Behavioral stress alters coronary vascular reactivity in borderline hypertensive rats," *Journal of Hypertension*, vol. 15, no. 3, pp. 301–307, 1997.

[147] A. D. Giulumian, S. G. Clark, and L. C. Fuchs, "Effect of behavioral stress on coronary artery relaxation altered with aging in BHR," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 276, no. 2, pp. R435–R440, 1999.

[148] M. Balkaya, V. Prinz, F. Custodis et al., "Stress worsens endothelial function and ischemic stroke via glucocorticoids," *Stroke*, vol. 42, no. 11, pp. 3258–3264, 2011.

[149] M. W. Radomski, R. M. J. Palmer, and S. Moncada, "Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 24, pp. 10043–10047, 1990.

[150] K. M. Rogers, C. A. Bonar, J. L. Estrella, and S. Yang, "Inhibitory effect of glucocorticoid on coronary artery endothelial function," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 283, no. 5, pp. H1922–H1928, 2002.

[151] T. Wallerath, K. Witte, S. C. Schäfer et al., "Down-regulation of the expression of endothelial NO synthase is likely to contribute to qlucocorticoid-mediated hypertension," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13357–13362, 1999.

[152] W. W. Simmons, D. Ungureanu-Longrois, G. K. Smith, T. W. Smith, and R. A. Kelly, "Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-arginine transport," *The Journal of Biological Chemistry*, vol. 271, no. 39, pp. 23928–23937, 1996.

[153] Y. Liu, D. Mladinov, J. L. Pietrusz, K. Usa, and M. Liang, "Glucocorticoid response elements and 11β-hydroxysteroid dehydrogenases in the regulation of endothelial nitric oxide synthase expression," *Cardiovascular Research*, vol. 81, no. 1, pp. 140–147, 2009.

[154] S. S. Jun, Z. Chen, M. C. Pace, and P. W. Shaul, "Glucocorticoids downregulate cyclooxygenase-1 gene expression and prostacyclin synthesis in fetal pulmonary artery endothelium," *Circulation Research*, vol. 84, no. 2, pp. 193–200, 1999.

[155] A. Zafir and N. Banu, "Induction of oxidative stress by restraint stress and corticosterone treatments in rats," *Indian Journal of Biochemistry and Biophysics*, vol. 46, no. 1, pp. 53–58, 2009.

[156] I. Y. Malyshev and E. B. Manukhina, "Stress, adaptation, and nitric oxide," *Biochemistry*, vol. 63, no. 7, pp. 840–853, 1998.

[157] G. B. Stefano, G. L. Fricchione, and T. Esch, "Relaxation: molecular and physiological significance," *Medical Science Monitor*, vol. 12, no. 9, pp. HY21–HY31, 2006.

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Research Article

Genotype-Related Effect of Crowding Stress on Blood Pressure and Vascular Function in Young Female Rats

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This study investigated the influence of chronic crowding stress on nitric oxide (NO) production, vascular function and oxidative status in young Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) female rats. Five-week old rats were exposed to crowding for two weeks. Crowding elevated plasma corticosterone ($P < 0.05$) and accelerated BP ($P < 0.01$ versus basal) only in BHR. NO production and superoxide concentration were significantly higher in the aortas of control BHR and SHR versus WKY. Total acetylcholine (ACh)-induced relaxation in the femoral artery was reduced in control SHR versus WKY and BHR, and stress did not affect it significantly in any genotype. The attenuation of ACh-induced relaxation in SHR versus WKY was associated with reduction of its NO-independent component. Crowding elevated NO production in all strains investigated but superoxide concentration was increased only in WKY, which resulted in reduced NO-dependent relaxation in WKY. In crowded BHR and SHR, superoxide concentration was either unchanged or reduced, respectively, but NO-dependent relaxation was unchanged in both BHR and SHR versus their respective control group. This study points to genotype-related differences in stress vulnerability in young female rats. The most pronounced negative influence of stress was observed in BHR despite preserved endothelial function.

1. Introduction

Stress is considered an etiological factor associated with the development of atherosclerosis and myocardial infarction as well as hypertension [1–3]. Research investigating mechanisms involved in stress-related cardiovascular disease and investigations concerning the roles of acute and chronic stress and their relation with the development of cardiovascular disease have attracted much attention [4]. However, despite this effort there are still ambiguous results concerning a causal association between stress and hypertension. Esler et al. [5, 6] concluded that mental stress is a cause of essential hypertension in humans; on the other hand, Sparrenberger et al. [7, 8] did not find psychosocial stress to be causally associated with hypertension. Discrepancies in results from human studies could arise from different stressors being considered, the intensity and duration of stress [9] and different populations investigated. Moreover, the age in which organism is exposed to aversive stimuli is another important factor. Regarding blood pressure (BP), many observations indicate that although the full manifestation of hypertension usually occurs later in life, its roots can be traced back to early ontogeny [10, 11]. One important period, common to both humans and rats, during which negative environmental factors affect later development of BP is the intrauterine period [11]. Additionally, data in animal studies indicate that the most important developmental window regarding BP after birth is between the sixth and eighth week of life in rats with a positive genetic predisposition to hypertension [12], that is, in the postweaning period, when rats have usually just been separated from the mother. The question, however, arises whether a given period of life might also be a critical window in subjects with a negative family history of hypertension or in those with a single hypertensive parent, and if stress acting at this age can accelerate an increase in their BP. As it is not possible to perform this kind of

study in humans (i.e., to intentionally expose children to chronic stress and to control the duration and intensity of the stressor), we simulated such an experiment in rats using normotensive Wistar-Kyoto (WKY, both parents normotensive), spontaneously hypertensive (SHR, both parents hypertensive), and borderline hypertensive (BHR, mother hypertensive and father normotensive) rats. We used crowding as a stressor acting in the postweaning period. Although crowding is a relatively mild stressor, it has a considerable effect on the hypothalamic-pituitary-adrenal (HPA) axis and the sympathoadrenal system in adult rats [13–15]. However, in addition to stress-activated systems there are several antistress systems including the L-arginine/nitric oxide (NO) system [16]. It has been shown that stress-related hormones and mediators may affect both vascular NO production and degradation, and thus its bioavailability [17]. We have shown previously that chronic crowding stress increased NO production in the aortas of adult normotensive WKY males, which was associated with improved acetylcholine-induced relaxation [18]. Thus, the L-arginine/NO system supposedly protects normotensive rats from developing stress-induced hypertension. However, elevated NO production per se might not imply better NO bioavailability, especially under conditions of oxidative stress, which was observed in hypertensive rats [19] as well during acute or chronic stress [15, 20–23]. Additionally, it is of interest that despite significant sex-related differences in cardiovascular regulation [24], NO production [25], and stress-induced neuroendocrine and cardiovascular responses [26, 27], the majority of experimental studies have used male rats.

Thus, this study investigated the influence of crowding in postweaning WKY, BHR, and SHR female rats on the development of BP and NO-mediated vascular function. We hypothesize that exposure to crowding stress in the postweaning period could accelerate the development of hypertension in rats with a genetic predisposition to hypertension by altering oxidative status and/or NO production in the vascular system.

2. Material and Methods

2.1. Animals and Treatment. All rats, WKY, BHR, and SHR, were born in our certified animal facility in order to retain the same environmental background for all the rats. BHR were F1 offspring of SHR dams and WKY sires. After birth, the rats were kept together with their mother until the end of the fifth week of life. Then they were separated from the mother, divided according to sex, and females were randomly divided into the control and crowding-exposed group. Control rats were kept under standard conditions (more than 200 cm^2/100 g of animal body weight) in groups of 4 rats per cage. Animals in the stress group were kept at five rats per cage and exposed to crowding stress, which was induced by reducing their living space to approximately 70 cm^2 per 100 g of animal weight for two weeks. The precise size of the cages was adjusted using special cages with one flexible wall. This wall was moved to the appropriate position on the same days as the body weights of the rats were determined. All animals were maintained at an ambient temperature of 22 to 24°C under artificial light with a 12 h light/dark cycle during the whole experiment. The rats were fed a standard pellet food and had water and food *ad libitum*. The experiments were performed in accordance with European Community and NIH guidelines for the use of experimental animals and were approved by the State Veterinary and Food Administration of the Slovak Republic.

2.2. Blood Pressure and Heart Rate Measurements. Systolic blood pressure (BP) and heart rate (HR) were measured indirectly by tail-cuff plethysmography between 08.00 and 11.00 a.m. as was described in detail previously [15]. In order to minimize the influence of nonspecific stress on BP measurements, all rats were handled and accustomed to the tail-cuff procedure before experimentation. Each value was calculated as the average of five measurements. BP and HR values were measured at the beginning of the experiment (basal) and after the 1st and 2nd weeks of the experiment. Body weight (BW) was determined on the same days.

2.3. Plasma Corticosterone Measurements. Rats were sacrificed by decapitation after brief CO_2 anesthesia between 07.30 and 09.30 a.m. [15]. Blood samples were collected in heparinized test tubes and immediately centrifuged at 850 g for 10 min at 4°C. Plasma samples were then stored at −80°C until analysis. Plasma corticosterone (pCort) was measured in duplicates using 20 μL of plasma with an enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA), according to the manufacturer's instructions.

2.4. Vascular Reactivity Measurements. Femoral arteries were carefully dissected, immersed, and transferred to cold physiological salt solution (PSS) and then cleaned to remove the adipose and connective tissues [15]. Arterial segments (1.0 to 1.5 mm long) were mounted in a small vessel wire myograph (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark). Dissection, mounting, and normalization of each vessel were performed according to [28]. Femoral artery reactivity measurements were performed as described previously [15]. Briefly, the experimental protocol consisted of the following steps. (1) PSS in the myograph chamber was changed to KPSS (i.e., PSS in which NaCl was exchanged for an equimolar concentration of KCl 125 mmol/L) and contraction was measured. (2) Ten μmol/L of norepinephrine (NE) was added and the contraction plateau value was recorded. (3) The artery was preconstricted with serotonin (1 μmol/L). When the contraction of the artery was steady, increasing concentrations of the vasodilator acetylcholine (ACh, from 0.001 to 10 μmol/L) were added in a cumulative manner and the endothelium-dependent relaxation response curve was recorded. (4) The same experiment was repeated after 25 min preincubation with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L) in the bath medium. (5) After serotonin preconstriction (1 μmol/L), the NO donor sodium nitroprusside ([29], SNP, 0.001 to 10 μmol/L) was added cumulatively and the relaxation response curve was recorded. (6) Finally, PSS was

changed to KPSS and, after reaching the contraction plateau, NE (10 μmol/L) was added and the maximal contraction of the artery was recorded. The artery was washed out four times with PSS and stabilized for 20 min after each step. All concentrations were expressed as final concentrations in the myograph chamber. All chemicals used were purchased from Sigma-Aldrich (Germany) and Merck Chemicals (Germany).

2.5. Nitric Oxide Synthase Activity. NO synthase (NOS) activity was determined by [^{3}H]-L-arginine conversion to [^{3}H]-L-citrulline (MP Biomedicals, USA) in crude tissue homogenates of aorta, as described previously [15] and was expressed as pmol/min/mg of protein.

2.6. Superoxide O_2^- Measurement. The assay was performed as described previously [30] with some modifications. The aortic rings were cut (10–15 mg), cleansed of connective tissue, and placed into ice cold PSS. Lucigenin (50 μmol/L) as well as tissue samples alone were added to PSS bubbled with pneumoxide (5% CO_2 and 95% O_2) at pH 7.4 and 37°C, and preincubated in the dark for 20 min. After preincubation, either background chemiluminescence or chemiluminescence produced by the aortic rings was measured for 6 min using a TriCarb 2910TR liquid scintillation analyzer (Perkin Elmer). Background counts were subtracted from values obtained from the samples. Results were expressed as counts per minute per mg of tissue (cpm/mg). All chemicals used were purchased from Sigma-Aldrich (Germany) and Merck Chemicals (Germany).

2.7. Statistical Analysis. Final body weight, heart rate and blood pressure, relative weight of the left ventricle and adrenal glands, normalized diameter, basal tension, and contraction data as well as the maximal response (E_{max}) and the concentration that produced a half maximal response (pD_2) were analyzed using two-way ANOVA and Bonferroni post-hoc test. Differences between concentration response curves were assessed with two-way repeated measures ANOVA and vertical contrast using Bonferroni adjustment. The SNP-induced vasorelaxant data were fitted by four-parameter logistic function using GraphPad Prism 5.0 (San Diego, CA, USA). To determine the depression in relaxation at higher concentrations of ACh in the ACh dose-response curves, the maximal response to ACh and the responses at subsequent ACh concentrations were compared using Dunnett's test (Figure 3). Because of the inherent non-normality of pCort data, these were analyzed using a generalized linear model (Gamma distribution, logarithmic link function in R v.2.15.0, http://www.r-project.org/). All values (except for pCort) are presented as mean ± SEM. pCort data are presented as mean ±95% confidence interval. The significance level of all tests was set at 5% ($\alpha = 0.05$).

3. Results

3.1. Basic Biometric, Cardiovascular, and Biochemical Parameters. There were significant differences in BW (Table 1) among the WKY, BHR, and SHR groups at the end of the experiment ($F_{2,71} = 5.7$; $P = 0.005$). The effect of age on BW was significant in all groups investigated (main effect of age $P < 0.001$). Stress reduced BW gain in general ($F_{1,71} = 27.8$, $P < 0.001$ main effect of stress); however, a significant stress-related reduction in BW gain was observed only in BHR and SHR versus the respective control (Table 1).

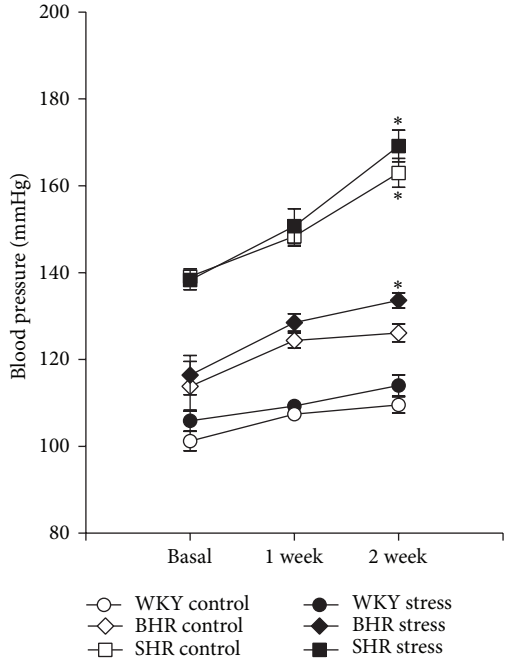

Figure 1: Effect of crowding stress on systolic blood pressure during two weeks of experiment. Data are presented as mean and SEM of 8–14 rats. $^*P < 0.05$ versus the basal value. Differences among genotypes were significant ($F_{2,71} = 29.0$; $P < 0.001$).

Basal BP in 5-week WKY, BHR, and SHR rats was 103 ± 2, 115 ± 4, and 138 ± 2 mmHg, respectively, and there were significant differences among the genotypes ($F_{2,71} = 29.0$; $P < 0.001$). As the postweaning period was associated with body growth there were also significant age-related changes in BP (main effect of age $P < 0.001$). Although stress did not affect BP significantly, there was a consistent trend toward increased BP in all crowded rats compared to controls (Figure 1). However, there was a significant effect of time ($F_{2,71} = 9.6$; $P < 0.001$) and a time-strain interaction ($F_{4,71} = 3.0$; $P = 0.023$). This analysis revealed nonsignificant time-related changes in BP in both WKY groups. In SHR, a significant elevation in BP was observed in both groups as compared to basal values ($P < 0.001$). In BHR, stress accelerated BP increase as opposed to the prestress value ($P = 0.034$), while no significant increase was seen in the control BHR (Figure 1). Regarding HR, there were significant genotype-related differences ($F_{2,71} = 13.2$, $P < 0.001$, main effect of genotype); however, age and stress failed to affect HR in all genotypes investigated (Table 1).

Plasma corticosterone concentration was significantly different among genotypes ($P = 0.014$), with the highest values observed in SHR rats (Table 1). Stress significantly increased pCort ($P = 0.027$, main effect of stress). Although

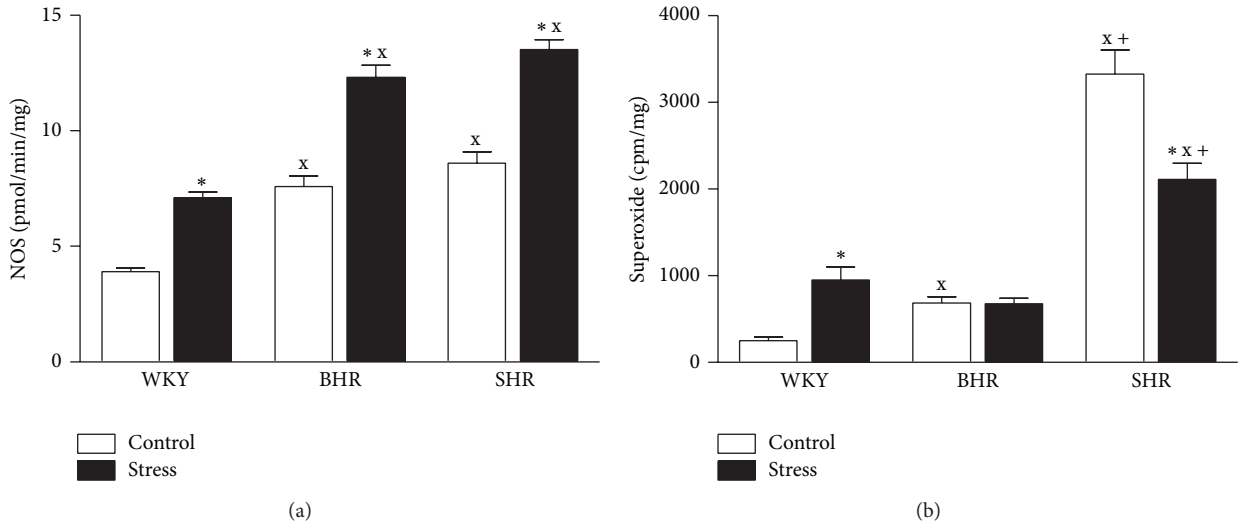

Figure 2: Effect of crowding stress on nitric oxide synthase (NOS) activity (a) and superoxide level (b) in the aortas of Wistar-Kyoto (WKY), borderline hypertensive (BHR), and spontaneously hypertensive (SHR) rats. $^{x}P < 0.05$ versus WKY respective group (control/stress); $^{+}P < 0.05$ versus BHR respective group (control/stress); $^{*}P < 0.05$ versus control group of the same genotype. Values represent mean ± SEM of six rats in each group.

Table 1: Basic biometric and cardiovascular parameters and plasma corticosterone levels of young female Wistar-Kyoto (WKY), borderline hypertensive (BHR), and spontaneously hypertensive (SHR) rats exposed to crowding.

	WKY		BHR		SHR	
	Control	Stress	Control	Stress	Control	Stress
BW basal (g)	107 ± 4	105 ± 5	87 ± 3	96 ± 3	78 ± 2^{x}	79 ± 3^{x+}
BW final (g)	158 ± 4	154 ± 4	159 ± 3	146 ± 4^{*}	121 ± 4^{x+}	112 ± 3^{x+}
BW gain (g)	51 ± 3	49 ± 4	73 ± 3^{x}	50 ± 2^{*}	43 ± 2^{+}	33 ± 2^{*x+}
Final HR (bpm)	422 ± 16	418 ± 11	440 ± 25	463 ± 14	497 ± 17^{x}	506 ± 31^{x}
LVM/BW (mg/g)	1.93 ± 0.07	2.08 ± 0.10	1.93 ± 0.06	2.12 ± 0.07	2.51 ± 0.07^{x+}	2.52 ± 0.07^{x+}
AG/BW (mg/g)	0.24 ± 0.01	0.25 ± 0.01	0.28 ± 0.01^{x}	0.28 ± 0.01^{x}	0.29 ± 0.01^{x}	0.29 ± 0.01^{x}
[1]pCort (ng/mL)	68 ± 25	76 ± 22	58 ± 17	393 ± 331^{x*}	243 ± 47^{x+}	287 ± 136^{x}

Values represent mean ± SEM (except for pCort) of 8–14 rats. BW: body weight; HR: heart rate; LVM/BW: left ventricular mass-to-body weight ratio; AG/BW: adrenal gland-to-body weight ratio; pCort: plasma corticosterone; $^{x}P < 0.05$ versus WKY respective group (control/stress); $^{+}P < 0.05$ versus BHR respective group (control/stress); $^{*}P < 0.05$ versus control group of the same genotype. [1]Because of the inherent non-normality of pCort, these data were analyzed using a generalized linear model (Gamma distribution, logarithmic link function) and they are presented as mean ± 95% confidence interval.

we observed higher levels of pCort in all WKY, BHR, and SHR stressed rats than in their respective control groups, the difference was statistically significant only in BHR. Additionally, significant genotype-related differences in relative adrenal gland weight (AG/BW) were observed ($F_{2,70} = 50.0$; $P < 0.001$). BHR and SHR, both the control and stress groups, had higher AG/BW ratios than the WKY groups but there was no significant effect of crowding (Table 1).

Under control conditions, relative left ventricular weights were significantly higher in SHR versus both WKY ($P < 0.001$) and BHR, respectively ($P < 0.02$), and no effect of crowding was observed (Table 1).

Genotype and crowding had significant effects on NO synthase activity in the aorta: $F_{2,30} = 18.2$, $P < 0.001$, main effect of genotype; and $F_{1,30} = 29.0$, $P < 0.001$, main effect of stress. NOS activity was elevated significantly in both control BHR and SHR versus WKY, and stress increased NO production significantly in all genotypes investigated (Figure 2(a)).

Superoxide levels were also increased in control BHR and SHR versus WKY ($F_{2,30} = 107$; $P < 0.001$, main effect of genotype). In crowded BHR and SHR, superoxide levels were either unchanged ($P = 0.72$) or reduced ($P < 0.001$), respectively, versus the respective control group; however, in SHR they were still significantly higher versus stressed WKY and BHR ($P < 0.05$ versus both) (Figure 2(b)).

3.2. Vascular Function. To investigate vascular function in the endothelium-intact femoral artery in individual genotypes as well as the effect of stress, we investigated normalized

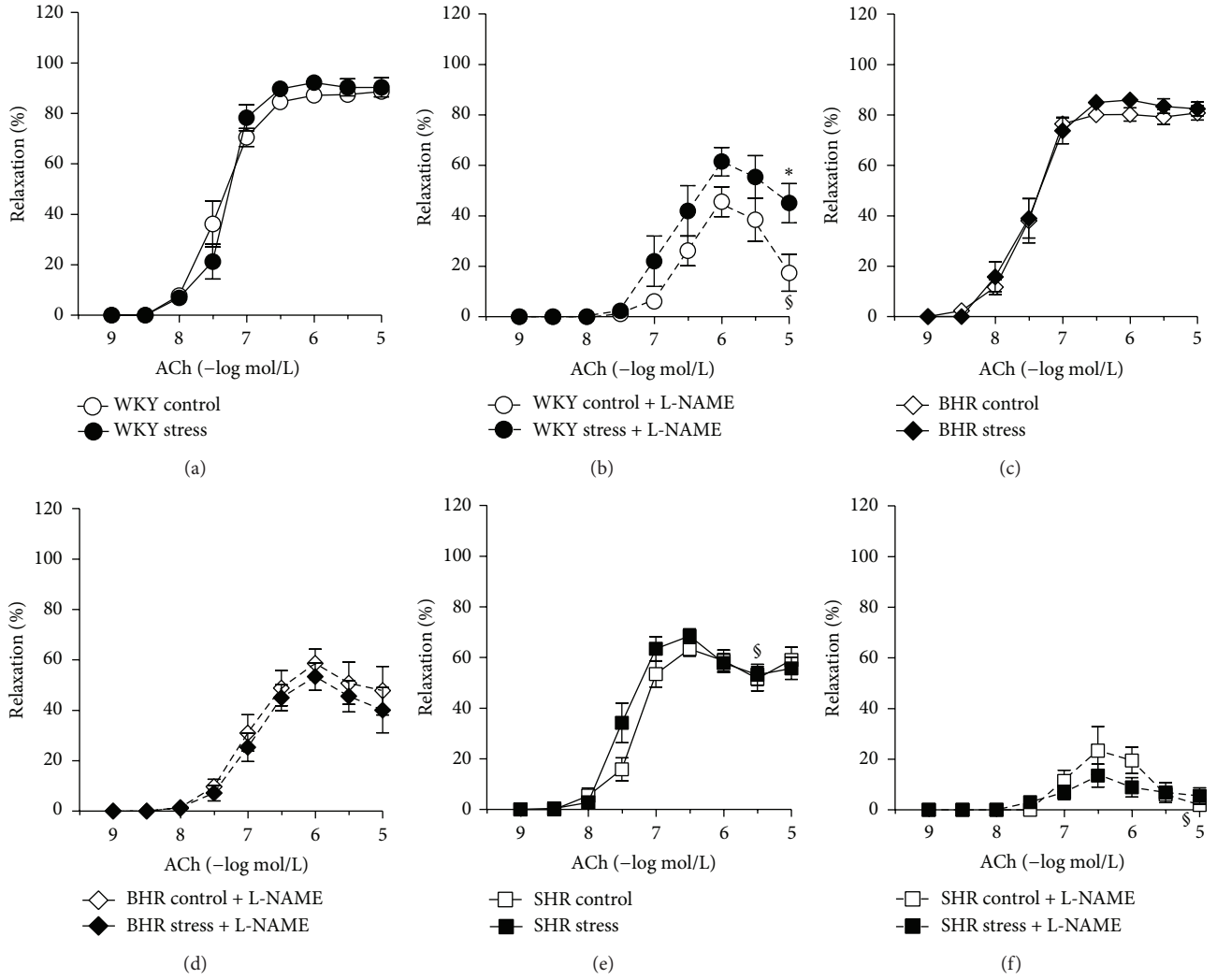

Figure 3: Effect of social stress on acetylcholine (ACh)-induced relaxation before ((a), (c), (e)) and after ((b), (d), (f)) pretreatment with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in the femoral arteries of young female Wistar-Kyoto (WKY), borderline hypertensive (BHR), and spontaneously hypertensive (SHR) rats. $^*P < 0.05$ compared to the respective value in control rats; $^\S P < 0.05$ compared to the maximal relaxation of the given group (indicating significant release of endothelium-derived contracting factors). Values represent mean ± SEM of 7–12 rats.

internal diameter and NE-, serotonin-, and KPSS-induced contractions as well as SNP-induced (Table 2) and ACh-induced relaxations (Table 2, Figures 3(a)–3(f)).

There were significant differences in the normalized diameter (ND) between BHR and SHR in both the control and stress groups, but crowding did not affect it significantly (Table 2). Norepinephrine and serotonin induced contractile responses in the femoral arteries. NE induced biphasic responses: a transient contraction (early response, phasic contraction) was followed by a depression in response almost to baseline value followed by a sustained contraction (delayed response, tonic contraction). There were differences among the rat strains in both phasic ($F_{2,48} = 10.6$; $P < 0.001$) and tonic ($F_{2,48} = 9.3$; $P < 0.001$) contractions, with significantly higher values observed in control BHR and SHR versus WKY. However, a significant effect of crowding was only observed in tonic contraction of BHR, which was attenuated versus control BHR (Table 2). On the other hand, no differences in serotonin-induced contractions were observed among the groups either before or after L-NAME pretreatment. The response of the arteries to serotonin after pretreatment with L-NAME was augmented compared to responses before L-NAME administration (main effect of L-NAME; $F_{1,92} = 25.7$; $P < 0.001$) and this effect was significant in both WKY groups as well as in the stressed BHR and SHR groups (Table 2).

Maximal KPSS-induced contraction (due to depolarization by K^+) was not altered by crowding in any genotype versus its respective control group (Table 2).

Cumulative addition of exogenous NO donor SNP affected vasorelaxation similarly in all groups investigated. There were no differences in SNP-E_{max} among the groups (Table 2). The sensitivity of the femoral arteries to SNP,

Table 2: Basic vascular parameters, constrictions, and relaxations of the femoral artery in young female Wistar-Kyoto (WKY), borderline hypertensive (BHR), and spontaneously hypertensive (SHR) rats exposed to crowding.

	WKY		BHR		SHR	
	Control	Stress	Control	Stress	Control	Stress
ND (μm)	579 ± 12	574 ± 15	630 ± 14	595 ± 10	537 ± 23^{+}	538 ± 11^{+}
Basal tension (kPa)	2.04 ± 0.21	1.92 ± 0.26	2.82 ± 0.20	2.24 ± 0.24	2.77 ± 0.53	2.84 ± 0.32
NE—phasic (kPa)	1.34 ± 0.27	2.42 ± 0.47	4.72 ± 0.64^{x}	3.75 ± 0.53	5.01 ± 1.05^{x}	5.75 ± 0.73^{x+}
NE—tonic (kPa)	0.15 ± 0.04	0.35 ± 0.15	6.36 ± 1.78^{x}	2.36 ± 0.63^{x*}	7.42 ± 2.02^{x}	7.14 ± 1.74^{x+}
Ser (kPa)	19.0 ± 0.6	19.9 ± 1.1	22.0 ± 1.5	23.7 ± 1.7	23.4 ± 1.7	21.9 ± 1.1
Ser—after L-NAME (kPa)	24.7 ± 0.5†	24.3 ± 1.7†	25.0 ± 1.8	28.9 ± 1.8†	27.7 ± 2.1	27.4 ± 1.3†
KPSS (kPa)	25.1 ± 1.2	25.0 ± 2.2	26.6 ± 2.7	32.6 ± 2.6^{x}	34.2 ± 2.8^{x+}	29.2 ± 2.2
SNP E_{max} (%)	99 ± 1.1	96 ± 1.3	98 ± 1.5	97 ± 2.0	98.0 ± 2.0	95 ± 2.3
SNP pD_2 (−log(mol/L))	7.96 ± 0.05	8.07 ± 0.06	8.09 ± 0.09	7.99 ± 0.11	7.69 ± 0.07^{x+}	7.63 ± 0.07^{x+}
ACh E_{max} (%)	88 ± 1.9	91 ± 1.8	81 ± 1.9	85 ± 2.1	58 ± 1.9^{x+}	60 ± 1.9^{x+}
ACh pD_2 (−log(mol/L))	7.39 ± 0.04	7.30 ± 0.03	7.50 ± 0.04	7.49 ± 0.05^{x}	7.36 ± 0.05	7.53 ± 0.05^{x}

Values represent mean ± SEM of 7–12 rats. ND: normalized diameter of the femoral artery at 13.3 kPa; NE: norepinephrine (10 μmol/L); Ser: serotonin (1 μmol/L); L-NAME: N^{G}-nitro-L-arginine methyl ester; KPSS: PSS in which NaCl was exchanged for an equimolar concentration of KCl; SNP: sodium nitroprusside; ACh: acetylcholine; $^{x}P < 0.05$ versus WKY respective group (control/stress); $^{+}P < 0.05$ versus BHR respective group (control/stress); $^{*}P < 0.05$ versus control group of the same genotype; $^{\dagger}P < 0.05$ versus the same group before L-NAME.

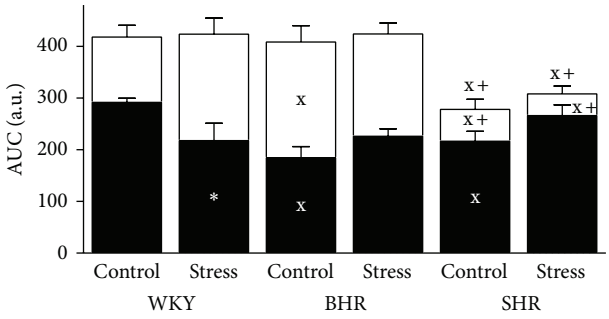

Figure 4: Effect of crowding stress on overall acetylcholine-induced relaxation and its NO-dependent (black bars) and NO-independent (white bars) components in the femoral arteries of Wistar-Kyoto (WKY), borderline hypertensive (BHR), and spontaneously hypertensive (SHR) rats. AUC—area under the curve; a.u.—arbitrary units; NO—nitric oxide; $^{x}P < 0.05$ versus WKY respective group (control/stress); $^{+}P < 0.05$ versus BHR respective group (control/stress); $^{*}P < 0.05$ versus control group of the same genotype. Marks above the columns denote differences in overall relaxation and marks in the white (black) part denote differences in NO-independent (NO-dependent) relaxation, respectively. Values represent mean ± SEM of 7–12 rats.

determined as pD_2, was significantly reduced in both SHR groups as compared to WKY and BHR (Table 2).

There were significant genotype-related differences in overall ACh-induced relaxation expressed as either ACh-E_{max} (Table 2, $F_{2,48} = 116$; $P < 0.001$) or the area under the ACh-induced dose-response curve (AUC) (Figure 4, $F_{2,48} = 27$; $P < 0.001$); significant reductions were observed in SHR versus both WKY and BHR ($P < 0.001$) for all comparisons. The main effect of stress on ACh-E_{max} and AUC was not significant. The sensitivity of the femoral arteries to ACh, measured as ACh-pD_2, was similar among the genotypes and stress failed to affect it compared to the control group of the same genotype. However, stress increased sensitivity to ACh in BHR and SHR versus stressed WKY (Table 2, $F_{2,48} = 3.9$; $P < 0.03$, effect of genotype-stress interaction).

Inhibition of NO synthase by L-NAME significantly reduced endothelium-dependent relaxations in all groups investigated (Figure 3). The NO-dependent component was reduced significantly in the young females with a genetic predisposition to high BP ($F_{2,48} = 3.2$; $P < 0.05$, main effect of genotype). On the other hand, the NO-independent component was significantly elevated in control BHR ($P < 0.01$) while a reduction was seen in control SHR versus WKY ($P < 0.05$; Figure 4). In addition, maximal relaxation after acute L-NAME pretreatment was significantly reduced in control SHR (27 ± 8%) versus WKY (47 ± 6%, $P < 0.05$).

There were significant differences in both the NO-dependent and NO-independent components of stressed WKY rats versus control (Figure 4). Crowding induced reduction of NO-dependent component of ACh-induced relaxation which was compensated by the elevation of NO-independent component in WKY, which was not observed in BHR and SHR (Figure 4).

4. Discussion

This study is the first to examine the effects of chronic stress on BP, superoxide, and NO production as well as vascular function in young normotensive, borderline hypertensive, and spontaneously hypertensive female rats in the postweaning period. Social stress produced by crowding, as described in this study, resulted in a generalized reduction in body weight gain as well as elevations in pCort and aortic NO production in all genotypes investigated. However, genotype-related differences in response to stress were observed in BP, superoxide concentration, and mechanism of vasorelaxation. Stress accelerated the increase in BP in BHR while no effect

was observed in WKY and SHR. Vascular studies revealed overall endothelial dysfunction only in SHR, while normal endothelial function was seen in BHR. However, reduced NO-dependent relaxation was observed in both control BHR and SHR versus WKY. Interestingly, stress elevated superoxide concentration and reduced NO-dependent relaxation only in WKY, which was not observed in rats with elevated blood pressure.

This study was established to determine if stress acting in the postweaning period (i.e., in the period shortly after separation from the mother) can accelerate BP increases in rats. This developmental period was chosen as it is a critical developmental window in SHR rats [10]. Additionally, chronic noise, which can be considered a stressor, increased BP in 3-4 year-old children in kindergarten [31], that is, the period when children are usually separated from their mothers for the first time in their lives. The above-mentioned study suggested that exposure to stressful stimuli during the period of life when children are learning to be without maternal protection might negatively affect cardiovascular regulation. In addition, stress was shown to alter body weight, which might be expected to differ depending on the stressor, in humans [32, 33] and rats [34, 35]. In this experimental study, reduced age-related body mass gain and a consistent trend toward increasing BP was seen in all stressed groups. However, the most pronounced effect of stress was observed in rats with one hypertensive parent (mother in this case), in which pCort, body mass gain, and BP were all significantly affected. In SHR, the age-related increase in BP was similar in the control and stressed groups, despite reduced growth, suggesting that in this strain their genetic predisposition to hypertension is the dominant factor and stress does not alter BP under the given conditions.

This study showed that crowding is a mild but effective chronic stress model in postweaning female rats, similar to what we have shown previously in adult normotensive males [15, 18]. However, in this study we observed significant genotype-related differences in the activation of the HPA axis as determined by pCort concentration. In agreement with previously published studies, we observed a higher basal corticosterone concentration in young SHR animals compared to the WKY strain [36] but not in BHR [37]. Regarding the effect of stress, the main effect of crowding on pCort concentration was significant, but the most pronounced increase was observed in BHR, suggesting that young females of this genotype are particularly sensitive to this stressor. Insignificant increases in pCort in WKY and SHR rats (versus their respective control) at the end of the experiment can be explained by better adaptation, which protects organisms against detrimental effects of chronic stress. Adaptation to chronic stress has been observed in various stress models in humans as well as animals [38, 39], while an inability to adapt may result in diseased states. Similar to pCort, stress-induced elevation in BP was significant only in BHR. In agreement with this, other studies have shown higher pressor responses in adult BHR males to various stressors as compared to WKY [40–42]. Interestingly, differences in pressor adaptation to stress were shown to be related to the central effect of corticosterone. Bechtold et al. [41] found that endogenous corticosterone acts via hindbrain glucocorticoid receptors (GR) to enhance the pressor response to stress in adult BHR males but promotes adaptation in WKY. Moreover, prenatal dexamethasone treatment (a synthetic glucocorticoid analogue) increased baseline arterial pressure selectively in BHR in both sexes, but pCort increased only in female BHR [37]. With respect to our results, we assume that a similar corticosterone-mediated mechanism could be responsible for the differential effects of crowding on BP development in young WKY and BHR females. But to the best of our knowledge, no information is available in the literature on the role of hindbrain GRs in stress-exposed SHR.

In addition to the pressor effects, corticosterone is involved in modulating body mass. A study by Akana et al. [43] revealed the narrow range of pCort (10–75 ng/mL) that is compatible with a normal growth rate. In their study, reduced body weight gain was observed in young normotensive male rats when pCort exceeded this range. Indeed, pCort levels in the above-mentioned interval, together with normal body weight gain, were observed in stressed WKY and control BHR rats in this study, while elevated pCort concentrations in stressed BHR as well as in both SHR groups were associated with reductions in body mass gain. Crowding has also been reported to reduce body weight gain [44] via reduced food intake in both sexes [44, 45], which was also seen in young females in this study (data not shown).

Furthermore, there are studies showing that glucocorticoids may alter vascular function, and hypertension was shown to be the most significant negative side effect of chronic glucocorticoid treatment in humans [46, 47]. Additionally, inhibition of glucocorticoid release prevented acute mental stress-induced endothelial dysfunction [48]. Glucocorticoids were shown to downregulate NO production by limiting tetrahydrobiopterin (BH_4) production [49] and eNOS gene transcription [50], which might result in endothelial dysfunction. Interestingly, in this experimental study, crowding did not affect maximal relaxation in any genotype, regardless of the pCort level, but it modified the mechanism of relaxation responses—its NO-dependent and independent components. The most pronounced alterations in the mechanism of vasorelaxation were observed in WKY, without alteration in BP, suggesting successful allostasis [51]. However, the absence of an influence of stress on overall relaxation in BHR (versus control), together with reduced NE-induced constriction, suggests that nonvascular mechanisms are involved in the acceleration of hypertension development seen in this genotype. In contrast, young control SHR females developed endothelial dysfunction, with reductions in both the NO-dependent and NO-independent components of relaxation (versus WKY), and stress failed to modify these changes. A finding of a reduced NO-independent component of ACh-induced relaxation in young SHR females is in agreement with our previous observation in adult SHR males [52]. In contrast, findings of a reduced NO-dependent component of ACh-induced relaxation in young control SHR and BHR females are in opposition with our recent observations of a positive correlation between BP and the NO-dependent component of relaxation in adult male BHR and SHR [52]. Whether this discrepancy results from

the animals' different ages, sexes, or both remains to be elucidated in further studies, yet methodological aspects can be excluded [23] as the same methods were used in both our studies.

Oxidative stress, which seems to play an important role in the development of endothelial dysfunction [23], is another parameter that was shown to be modulated by glucocorticoids [50]. In this study, elevated superoxide concentration in the aorta was observed under control conditions in both genotypes with a predisposition to hypertension; however, it was more pronounced in SHR than in BHR. Regarding crowding stress, it elevated superoxide in WKY, in agreement with studies that found oxidative damage in normotensive rats exposed to chronic stress [22, 53, 54]. Interestingly, crowding stress had no effect in BHR and yet reduced superoxide concentration in SHR versus its respective control group. However, the level of superoxide in stressed SHR was still approximately two-fold higher compared to WKY and BHR under both control and stress conditions. The reason for this difference is unknown but we assume it may result from genotype-related differences in antioxidant defense systems. Indeed, we found elevations in plasma superoxide dismutase (SOD) activity and reduced lipoperoxide formation in blood as well as increased expression of SOD 1 and 2 in the kidneys of both BHR and SHR females exposed to stress, which was not observed in WKY (data not shown). However, neither elevated vascular NO synthase activity, in association with reduced superoxide level, was able to significantly improve NO-dependent relaxation in stressed SHR, which may be related to guanylate cyclase desensitization [55].

Regarding control SHR, despite enormous research, the exact mechanism of initiation of hypertension in this strain is still unclear. Our data suggest that elevated BP might be associated with alterations in corticosterone concentration or signaling and/or increased superoxide levels which might further lead to sympathetic activation and acceleration of the renin-angiotensin-aldosterone system [56, 57] and thus to an accentuated release of reactive oxygen species (ROS). Enormous ROS production can be implicated in the increased release of the endothelium-derived contracting factors, which may play a crucial role in the development of NO-independent endothelial dysfunction in genetic hypertension [58]. Moreover, possible structural changes in the vasculature of SHR and altered cell-to-cell communication may both participate in altered vascular function observed in SHR rats.

All together, these alterations may damage endothelial function, despite elevated vascular NO production, which would lead to increased peripheral resistance and the development of hypertension disease. If such is the case, how might the elevated BP in control BHR be explained when its basal corticosterone level was similar to that in WKY? It might be explained by elevated sensitivity to everyday stressors, which can be associated with transient periods of high corticosterone levels even under control conditions. As these periods were interrupted by periods of relief, the elevation of ROS could be relatively mild. Under such conditions the development of endothelial dysfunction would be delayed while the central effects of corticosterone acting via GRs might be responsible for the mild increase in BP observed in BHR. In addition, disruption or resetting of the baroreflex [42] may also participate in the gradual increase of BP in BHR. In such a situation, exposure of BHR to chronic social stress, associated with long-term HPA activation and imbalance in sympathovagal control [42], could accelerate the increase in BP as was observed in this study. The absence of signs of endothelial dysfunction in young BHR females exposed to crowding might also be explained by the relatively short duration and intensity of the stress used in this study, which may not be sufficient to exceed the adaptive mechanisms protecting vascular function, such as elevated NO production and reduced NE-constriction. Reduced NE-induced constriction in BHR may result from augmented sensitivity and number of β-2 adrenergic receptors due to elevated corticosterone [59] which may blunt α-1 adrenergic receptor-mediated constriction. Yet prolongation of stress could be associated with later development of NO-independent endothelial dysfunction, as we observed previously in WKY males exposed to 12 weeks of crowding [15]. Another possible explanation for the lack of stress-induced endothelial dysfunction is that the increasing estrogenic activity of young females at this age protects them from its development [60, 61].

5. Conclusions

In conclusion, this study resulted in several important findings. First of all, it points out that rats without a predisposition to hypertension were able to adapt to the given stressor via successful allostasis. Second, we showed significantly higher corticosterone and superoxide concentrations in young SHR females, which may play a role in the early development of endothelial dysfunction and hypertension in this strain. Furthermore, this study showed that the genetic predisposition to hypertension in SHR is the dominant factor in hypertension developing and stress did not aggravate the increase in BP under the given conditions. Additionally, we confirmed an elevation in vascular NO production after chronic stress exposure, which can act as an antistress mechanism in young females. However, the most important result of this study is that exposure to stress during a sensitive developmental period (in rats between the fifth and seventh weeks of life) can accelerate the inevitable increase in BP in juvenile females that are the offspring of hypertensive mothers and normotensive fathers. These females were more vulnerable to stress than female offspring of two normotensive or two hypertensive parents. Data suggested that their susceptibility to stress-induced hypertension resulted from the central effects of corticosterone rather than from altered vascular function.

On balance then, this study suggests caution in chronic exposure to stressors in childhood and juvenescence, especially in individuals where one parent suffers from hypertension. Although the progress of hypertension is usually slow and the disease is fully manifested in adulthood, it might originate from stressful environmental conditions in early periods of life.

Conflict of Interests

The authors declare that they have no conflicts of interests.

Authors' Contribution

Peter Slezak and Angelika Puzserova contributed to the same extent.

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References

[1] C. N. B. Merz, J. Dwyer, C. K. Nordstrom, K. G. Walton, J. W. Salerno, and R. H. Schneider, "Psychosocial stress and cardiovascular disease: pathophysiological links," *Behavioral Medicine*, vol. 27, no. 4, pp. 141–147, 2002.

[2] M. S. Kopp and J. Réthelyi, "Where psychology meets physiology: chronic stress and premature mortality—the Central-Eastern European health paradox," *Brain Research Bulletin*, vol. 62, no. 5, pp. 351–367, 2004.

[3] A. Rosengren, S. Hawken, S. Ôunpuu et al., "Association of psychosocial risk factors with risk of acute myocardial infarction in 11 119 cases and 13 648 controls from 52 countries (the INTERHEART study): case-control study," *The Lancet*, vol. 364, no. 9438, pp. 953–962, 2004.

[4] A. Steptoe, A. Rosengren, and P. Hjemdahl, "Introduction to cardiovascular disease, stress and adaptation," in *Stress and Cardiovascular Diseases*, P. Hjemdahl, A. Rosengren, and A. Steptoe, Eds., pp. 1–14, Springer, 2012.

[5] M. Esler, N. Eikelis, M. Schlaich et al., "Chronic mental stress is a cause of essential hypertension: presence of biological markers of stress," *Clinical and Experimental Pharmacology and Physiology*, vol. 35, no. 4, pp. 498–502, 2008.

[6] M. Esler, "The causal role of chronic mental stress in the pathogenesis of essential hypertension," in *Stress and Cardiovascular Disease*, P. Hjemdahl, A. Rosengren, and A. Steptoe, Eds., pp. 273–283, Springer, 2012.

[7] F. Sparrenberger, S. C. Fuchs, L. B. Moreira, and F. D. Fuchs, "Stressful life events and current psychological distress are associated with self-reported hypertension but not with true hypertension: results from a cross-sectional population-based study," *BMC Public Health*, vol. 8, article 357, 2008.

[8] F. Sparrenberger, F. T. Cichelero, A. M. Ascoli et al., "Does psychosocial stress cause hypertension? A systematic review of observational studies," *Journal of Human Hypertension*, vol. 23, no. 1, pp. 12–19, 2009.

[9] D. L. Wong, T. C. Tai, D. C. Wong-Faull et al., "Epinephrine: a short- and long-term regulator of stress and development of illness—a potential new role for epinephrine in stress," *Cellular and Molecular Neurobiology*, vol. 32, no. 5, pp. 737–748, 2012.

[10] J. Zicha and J. Kuneš, "Ontogenetic aspects of hypertension development: analysis in the rat," *Physiological Reviews*, vol. 79, no. 4, pp. 1227–1282, 1999.

[11] J. Kunes, M. Kadlecova, I. Vaneckova et al., "Critical developmental periods in the pathogenesis of hypertension," *Physiological Research*, vol. 61, supplement 1, pp. S9–17, 2012.

[12] J. Kuneš and J. Zicha, "Developmental windows and environment as important factors in the expression of genetic information: a cardiovascular physiologist's view," *Clinical Science*, vol. 111, no. 5, pp. 295–305, 2006.

[13] J. Bugajski, "Social stress adapts signaling pathways involved in stimulation of the hypothalamic-pituitary-adrenal axis," *Journal of Physiology and Pharmacology*, vol. 50, no. 3, pp. 367–379, 1999.

[14] J. Djordjević, G. Cvijić, and V. Davidović, "Different activation of ACTH and corticosterone release in response to various stressors in rats," *Physiological Research*, vol. 52, no. 1, pp. 67–72, 2003.

[15] A. Puzserova, P. Slezak, P. Balis et al., "Long-term social stress induces nitric oxide-independent endothelial dysfunction in normotensive rats," *Stress*, vol. 16, pp. 331–339, 2013.

[16] O. N. Bondarenko, N. A. Bondarenko, I. Y. Malyshev, and E. B. Manukhina, "Antistress effect of nitric oxide," *Biology Bulletin*, vol. 28, no. 4, pp. 387–393, 2001.

[17] N. Toda and M. Nakanishi-Toda, "How mental stress affects endothelial function," *Pflügers Archiv*, vol. 462, no. 6, pp. 779–794, 2011.

[18] I. Bernátová, A. Púzserová, J. Navarová, Z. Csizmadiová, and M. Zeman, "Crowding-induced alterations in vascular system of Wistar-Kyoto rats: role of nitric oxide," *Physiological Research*, vol. 56, no. 5, pp. 667–669, 2007.

[19] S. Grunfeld, C. A. Hamilton, S. Mesaros et al., "Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats," *Hypertension*, vol. 26, no. 6, pp. 854–857, 1995.

[20] A. Zafir and N. Banu, "Modulation of in vivo oxidative status by exogenous corticosterone and restraint stress in rats," *Stress*, vol. 12, no. 2, pp. 167–177, 2009.

[21] S. Kwiecień, M. W. Pawlik, T. Brzozowski et al., "Nitric oxide (NO)-releasing aspirin and (NO) donors in protection of gastric mucosa against stress," *Journal of Physiology and Pharmacology*, vol. 59, no. 2, pp. 103–115, 2008.

[22] E. F. Kamper, A. Chatzigeorgiou, O. Tsimpoukidi et al., "Sex differences in oxidant/antioxidant balance under a chronic mild stress regime," *Physiology and Behavior*, vol. 98, no. 1-2, pp. 215–222, 2009.

[23] I. Bernatova, M. Victoria Conde, J. Kopincova, M. Carmen González, A. Puzserova, and S. M. Arribas, "Endothelial dysfunction in spontaneously hypertensive rats: focus on methodological aspects," *Journal of Hypertension*, vol. 27, no. 6, pp. S27–S31, 2009.

[24] A. M. Dart, X.-J. Du, and B. A. Kingwell, "Gender, sex hormones and autonomic nervous control of the cardiovascular system," *Cardiovascular Research*, vol. 53, no. 3, pp. 678–687, 2002.

[25] O. Semyachkina-Glushkovskaya, T. Anishchenko, S. Kapralov et al., "Sex differences in cardiovascular control by nitric oxide in normotensive and hypertensive rats," *Health*, vol. 2, pp. 897–905, 2010.

[26] B. M. Kudielka and C. Kirschbaum, "Sex differences in HPA axis responses to stress: a review," *Biological Psychology*, vol. 69, no. 1, pp. 113–132, 2005.

[27] T. G. Anishchenko, O. V. Glushkovskaya-Semyachkina, V. A. Berdnikova, and T. A. Sindyakova, "Sex-related differences in cardiovascular stress reactivity in healthy and hypertensive rats," *Bulletin of Experimental Biology and Medicine*, vol. 143, no. 2, pp. 178–181, 2007.

[28] M. J. Mulvany, "Procedures for investigation of small vessels using small vessel myograph," Danish Myo Technology, 16-8-2012, 2004, http://www.dmt.dk/files/manualer/procedures_for_investigation.pdf.

[29] F. C. Munhoz, S. R. Potje, A. C. Pereira et al., "Hypotensive and vasorelaxing effects of the new NO-donor [Ru(terpy)(bdq)NO^+]$^{3+}$ in spontaneously hypertensive rats," *Nitric Oxide*, vol. 26, no. 2, pp. 111–117, 2012.

[30] T. G. Guzik and K. M. Channon, "Measurement of vascular reactive oxygen species production by chemiluminescence," in *Hypertension: Methods and Protocols*, J. P. Fennell and A. H. Baker, Eds., pp. 73–89, Springer, Humana Press, Totowa, NJ, USA, 2005.

[31] V. Regecova and E. Kellerova, "Effects of urban noise pollution on blood pressure and heart rate in preschool children," *Journal of Hypertension*, vol. 13, no. 4, pp. 405–412, 1995.

[32] A. L. Miller, C. Clifford, J. Sturza et al., "Blunted cortisol response to stress is associated with higher body mass index in low-income preschool-aged children," *Psychoneuroendocrinology*, vol. 38, pp. 2611–2617, 2013.

[33] R. G. Kuijer and J. A. Boyce, "Emotional eating and its effect on eating behaviour after a natural disaster," *Appetite*, vol. 58, no. 3, pp. 936–939, 2012.

[34] L. Bobrovskaya, D. Beard, E. Bondarenko et al., "Does exposure to chronic stress influence blood pressure in rats?" *Autonomic Neuroscience*, vol. 177, pp. 217–223, 2013.

[35] B. M. Sweis, K. K. Veverka, E. S. Dhillon et al., "Individual differences in the effects of chronic stress on memory: behavioral and neurochemical correlates of resiliency," *Neuroscience*, vol. 246, pp. 142–159, 2013.

[36] T.-L. Sterley, F. M. Howells, and V. A. Russell, "Effects of early life trauma are dependent on genetic predisposition: a rat study," *Behavioral and Brain Functions*, vol. 7, article 11, 2011.

[37] A. G. Bechtold, K. Vernon, T. Hines, and D. A. Scheuer, "Genetic predisposition to hypertension sensitizes borderline hypertensive rats to the hypertensive effects of prenatal glucocorticoid exposure," *Journal of Physiology*, vol. 586, no. 2, pp. 673–684, 2008.

[38] N. Grissom and S. Bhatnagar, "Habituation to repeated stress: get used to it," *Neurobiology of Learning and Memory*, vol. 92, no. 2, pp. 215–224, 2009.

[39] J. G. Tasker and J. P. Herman, "Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis," *Stress*, vol. 14, no. 4, pp. 398–406, 2011.

[40] I. Bernatova, Z. Csizmadiova, J. Kopincova, and A. Puzserova, "Vascular function and nitric oxide production in chronic social-stress-exposed rats with various family history of hypertension," *Journal of Physiology and Pharmacology*, vol. 58, no. 3, pp. 487–501, 2007.

[41] A. G. Bechtold, G. Patel, G. Hochhaus, and D. A. Scheuer, "Chronic blockade of hindbrain glucocorticoid receptors reduces blood pressure responses to novel stress and attenuates adaptation to repeated stress," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 296, no. 5, pp. R1445–R1454, 2009.

[42] O. Šarenac, M. Lozić, S. Drakulić et al., "Autonomic mechanisms underpinning the stress response in borderline hypertensive rats," *Experimental Physiology*, vol. 96, no. 6, pp. 574–589, 2011.

[43] S. F. Akana, C. S. Cascio, J. Shinsako, and M. F. Dallman, "Corticosterone: narrow range required for normal body and thymus weight and ACTH," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 249, no. 5, pp. R527–R532, 1985.

[44] H. S. Nagaraja and P. S. Jeganathan, "Voluntary alcohol drinking & caloric intake in rats exposed to crowding stress," *Indian Journal of Medical Research*, vol. 116, pp. 111–116, 2002.

[45] K. J. Brown and N. E. Grunberg, "Effects of environmental conditions on food consumption in female and male rats," *Physiology and Behavior*, vol. 60, no. 1, pp. 293–297, 1996.

[46] J. E. Goodwin and D. S. Geller, "Glucocorticoid-induced hypertension," *Pediatric Nephrology*, vol. 27, no. 7, pp. 1059–1066, 2012.

[47] C. G. Schnackenberg, M. H. Costell, D. J. Krosky et al., "Chronic inhibition of 11β-hydroxysteroid dehydrogenase type 1 activity decreases hypertension, insulin resistance, and hypertriglyceridemia in metabolic syndrome," *BioMed Research International*, vol. 2013, Article ID 427640, 10 pages, 2013.

[48] A. J. M. Broadley, A. Korszun, E. Abdelaal et al., "Inhibition of cortisol production with metyrapone prevents mental stress-induced endothelial dysfunction and baroreflex impairment," *Journal of the American College of Cardiology*, vol. 46, no. 2, pp. 344–350, 2005.

[49] W. W. Simmons, D. Ungureanu-Longrois, G. K. Smith, T. W. Smith, and R. A. Kelly, "Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-arginine transport," *Journal of Biological Chemistry*, vol. 271, no. 39, pp. 23928–23937, 1996.

[50] S. P. Duckles and V. M. Miller, "Hormonal modulation of endothelial NO production," *Pflügers Archiv*, vol. 459, no. 6, pp. 841–851, 2010.

[51] R. A. Sarabdjitsingh, M. Joëls, and E. R. de Kloet, "Glucocorticoid pulsatility and rapid corticosteroid actions in the central stress response," *Physiology and Behavior*, vol. 106, no. 1, pp. 73–80, 2012.

[52] A. Puzserova, J. Kopincova, P. Slezak et al., "Endothelial dysfunction in femoral artery of the hypertensive rats is nitric oxide independent," *Physiological Research*, vol. 62, pp. 615–629, 2013.

[53] M. Devaki, R. Nirupama, and H. N. Yajurvedi, "Chronic stress-induced oxidative damage and hyperlipidemia are accompanied by atherosclerotic development in rats," *Stress*, vol. 16, pp. 233–243, 2013.

[54] A. Zafir and N. Banu, "Induction of oxidative stress by restraint stress and corticosterone treatments in rats," *Indian Journal of Biochemistry and Biophysics*, vol. 46, no. 1, pp. 53–58, 2009.

[55] A. Friebe and D. Koesling, "Regulation of nitric oxide-sensitive guanylyl cyclase," *Circulation Research*, vol. 93, no. 2, pp. 96–105, 2003.

[56] H. Kobori, M. Nangaku, L. G. Navar, and A. Nishiyama, "The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease," *Pharmacological Reviews*, vol. 59, no. 3, pp. 251–287, 2007.

[57] D. A. Scheuer and A. G. Bechtold, "Glucocorticoids potentiate central actions of angiotensin to increase arterial pressure," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 280, no. 6, pp. R1719–R1726, 2001.

[58] I. Bernatova, "Endothelial dysfunction in experimental models of arterial hypertension: cause or consequence?" *BioMed Research International*. In press.

[59] A. Jazayeri and W. J. Meyer III, "Glucocorticoid modulation of β-adrenergic receptors of cultured rat arterial smooth muscle cells," *Hypertension*, vol. 12, no. 4, pp. 393–398, 1988.

[60] G. S. Ceravolo, F. P. Filgueira, T. J. Costa et al., "Conjugated equine estrogen treatment corrected the exacerbated aorta oxidative stress in ovariectomized spontaneously hypertensive rats," *Steroids*, vol. 78, pp. 341–346, 2013.

[61] L. Brandin, G. Bergström, K. Manhem, and H. Gustafsson, "Oestrogen modulates vascular adrenergic reactivity of the spontaneously hypertensive rat," *Journal of Hypertension*, vol. 21, no. 9, pp. 1695–1702, 2003.

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Review Article

Nitric Oxide, Oxidative Stress, and p66^{Shc} Interplay in Diabetic Endothelial Dysfunction

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Increased oxidative stress and reduced nitric oxide (NO) bioavailability play a causal role in endothelial cell dysfunction occurring in the vasculature of diabetic patients. In this review, we summarized the molecular mechanisms underpinning diabetic endothelial and vascular dysfunction. In particular, we focused our attention on the complex interplay existing among NO, reactive oxygen species (ROS), and one crucial regulator of intracellular ROS production, p66^{Shc} protein.

1. Introduction

Endothelial cells (ECs) synthesize and release different molecules that orchestrate metabolic, vascular, and cellular responses. Among them, nitric oxide (NO) is a key regulatory molecule of paramount importance for endothelial function and vascular tone relaxation [1, 2]. Notably, reduced endothelial cell nitric oxide synthase (eNOS) expression and/or NO bioavailability are associated with decreased EC survival and with endothelial dysfunction [3]. Indeed, a dysfunctional endothelium is not able to oppose vasoconstrictor stimuli, causing the increase of the arterial tone.

Reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radicals, also play a pivotal role in endothelial and vascular function as well as in vascular tone constriction [4]. ROS are generated as a consequence of aerobic metabolism and are produced by several cellular sources: plasma membrane NADPH oxidase (NOX), mitochondria, and different enzymes, such as several oxidases, peroxidases, cytochromes, mono- and dioxygenases, and uncoupled NOS.

The amount of ROS within the cell is finely modulated by enzymatic and nonenzymatic antioxidant defenses such as superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (GPx), and glutathione. Physiological ROS levels play an important role as second messengers within the intracellular signaling. Indeed, ROS can be actively generated and mediate physiological intracellular signalling as second messengers [5]. However, ROS production exacerbation or insufficient scavenging has been demonstrated to impair many biological processes including endothelial function in several pathological contexts.

A strict link exists between NOS activity and ROS production, since NOS uncoupling leads to the production of superoxide anion rather than NO. One of the major determinants of NOS uncoupling is the bioavailability of the cofactor tetrahydrobiopterin (BH_4) [6]. ROS as well as peroxynitrite ($ONOO^-$), another potent oxidant produced by the reaction of superoxide anion with NO, induce BH_4 degradation leading to eNOS uncoupling and to a reduction of the amount of endothelium-derived NO that is required for vascular relaxation and EC survival and proliferation [7].

The cellular pathways induced by ROS increase are known to provoke growth arrest and senescence, as well as cell death, either by apoptosis or by necrosis, according to the level of oxidative stress experienced by the cell, its genotype,

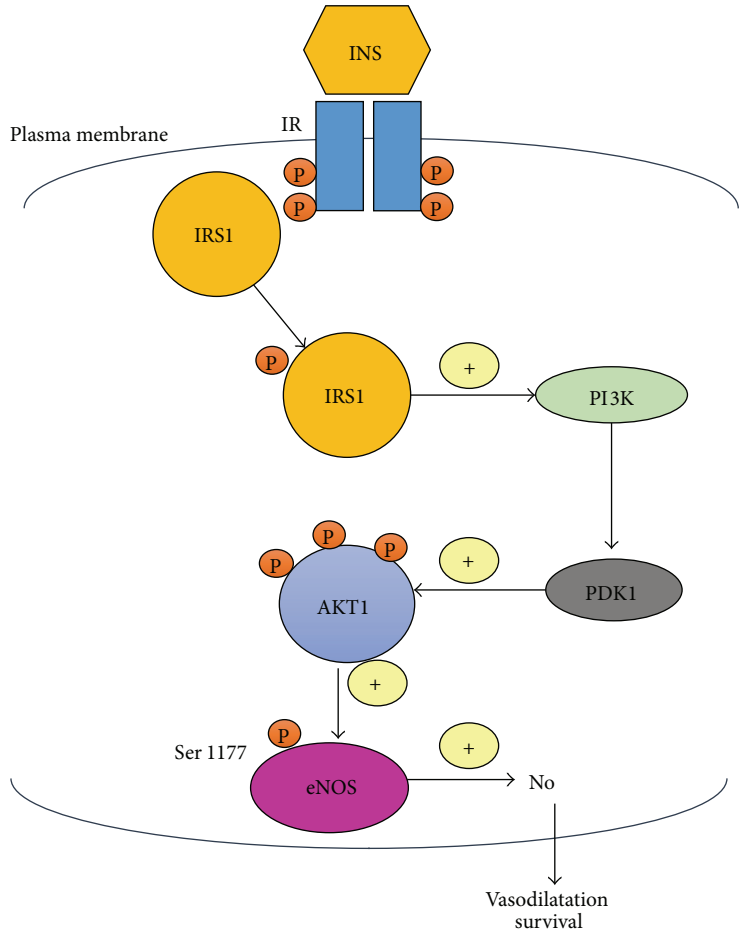

Figure 1: Intracellular insulin pathway. Insulin (INS), upon binding to its receptor, activates the insulin receptor tyrosine kinase, inducing tyrosine phosphorylation of the insulin receptor substrate-1 (IRS1). Phosphorylated IRS1 binds and activates phosphoinositol 3-kinase (PI3K), leading to the activation of serine-kinase phosphoinositide-dependent kinase 1 (PDK1), which activates AKT1. AKT1 phosphorylates eNOS at Ser-1177, leading to increased activity of eNOS and production of NO, which induces vasodilatation and EC survival. This pathway is strongly compromised in insulin resistance and diabetes mellitus.

and a multitude of epigenetic changes [8, 9]. A pivotal role in ROS-induced apoptosis is played by the p66 isoform of ShcA protein ($p66^{Shc}$), a fundamental regulator of mitochondrial ROS production by a variety of different stimuli [10]. Moreover, a fundamental role played by microRNAs is emerging [11, 12], indicating that noncoding RNAs play a major role in the establishment of pathological conditions associated with ROS imbalance, including diabetes mellitus [13–15].

In this review, we will focus our attention on the mechanisms regulating the correct balance and the complex interplay among ROS, NO, and $p66^{Shc}$ that are crucial for EC function. We will also show how the alteration of this network is one of the driving pathogenetic mechanisms underpinning diabetic vasculopathy and endothelial dysfunction.

2. Endothelial Dysfunction in Diabetes Mellitus

2.1. NO Bioavailability Reduction and ROS Increase. The regulation of NO metabolism is particularly important in diabetes mellitus, since the activation of eNOS has been demonstrated to be under the insulin control [16–18]. In particular, it has been shown that insulin (INS) binding to its receptor activates the insulin receptor tyrosine kinase activity, resulting in tyrosine phosphorylation of the insulin receptor substrate-1 (IRS1). Phosphorylated IRS1 binds and activates phosphoinositol 3-kinase (PI3 K), leading to activation of serine-kinase phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates and activates v-akt murine thymoma viral oncogene homolog 1 (AKT1). In turn, AKT1 directly phosphorylates eNOS at Ser-1177, leading to increased activity of eNOS and production of NO (Figure 1). Accordingly, IRS-1 mutations in ECs decrease insulin-stimulated eNOS phosphorylation and eNOS gene expression [19] and knockout mice of the endothelial-specific insulin receptor display decreased eNOS expression and endothelial vasodilator function impairment [20]. Moreover, animal models of insulin resistance, including the obese Zucker rat, display defects in the PI3 kinase/AKT1 system and impaired NO bioavailability [21].

Another signaling pathway that is activated by insulin in diabetes is the mitogen-activated protein kinases (MAPK)

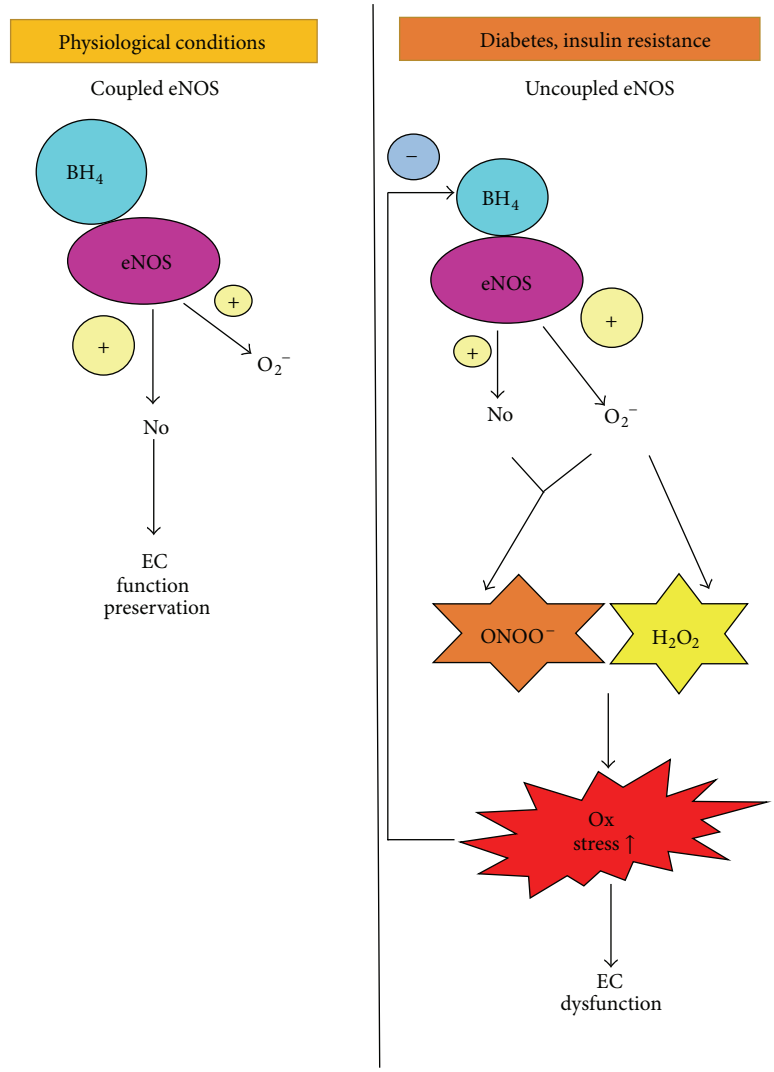

FIGURE 2: eNOS uncoupling in diabetes mellitus. Diabetes and insulin resistance are associated with eNOS uncoupling due to decreased levels of BH_4. eNOS uncoupling leads to the production of superoxide anion (O_2^-), rather than NO. Superoxide, in turn, is dismutated to H_2O_2 or reacts with NO, leading to the formation of peroxynitrite ($ONOO^-$) and to a further it decreases NO bioavailability. Increased $ONOO^-$ and H_2O_2 levels induce oxidative stress that further aggravates BH_4 depauperation.

pathway viathe small GTPase Ras [22]. The Ras/MAPK insulin-signaling pathway generally leads to cellular growth and proliferation. In ECs, activation of this pathway has been linked to the expression of endothelin-1, which is a potent mitogen and vasoconstrictor, and to the expression of proinflammatory adhesion molecules such as ICAM-1 [23]. In diabetes mellitus and insulin resistance, insulin-mediated activation of eNOS viaPI3 kinase/AKT1 is inhibited, while the adverse effects of insulin remain unopposed, which may promote vascular disease [24]. Blood flow and other physiological stimuli activate eNOS viaPI3 kinase/AKT1; therefore, an impairment of this signaling mechanism in diabetes may have broad implications for vascular dysfunction.

NOX is a membrane associated multisubunit complex that generates superoxide anion and is involved in the oxidative burst of inflammatory cells as well as in EC signaling [25]. In pathological conditions, including diabetes mellitus, NOX activity and superoxide production are increased [26, 27]. Increased free fatty acid concentration activates NOX and the proinflammatory transcription factor NFκB [28]. Moreover, NOX expression is upregulated by the systemic vasoconstrictor angiotensin II [29] that is increased in type 2 diabetic patients or animal models, along with its generating enzymes and receptors. Moreover, angiotensin II seems to play a role in the regulation of insulin secretion by pancreatic beta-cells and insulin sensitivity by peripheral tissues, which are two critical factors contributing to the development of type 2 diabetes. Accordingly, angiotensin converting enzymes inhibitors show positive vascular effects in diabetes [30, 31].

Finally, diabetes is associated with eNOS uncoupling and decreased BH_4 levels and therefore increased ROS production (Figure 2). In keeping with these data, BH_4 supplementation improves NO production and endothelial function both

in experimental diabetes models [32] and in human subjects with type 2 diabetes mellitus [33, 34].

Interestingly, Thomas et al. reported the effects of H_2O_2 treatment in ECs on eNOS phosphorylation. It was shown that a short H_2O_2 treatment with 300 μM H_2O_2 induced promotion of eNOS activity and modulation of the eNOS phosphorylation status at Ser-1177 in porcine aortic endothelial cells via a calcium- and AKT-dependent pathway [35]. Additionally, another study demonstrated that eNOS mRNA expression was increased in bovine aortic ECs treated with 100 μM H_2O_2 for 24 hours [36].

Most probably, both these effects might represent adaptive responses of ECs to maintain NO bioactivity under conditions of enhanced oxidative stress. On the other hand, a very interesting report demonstrated that human eNOS phosphorylation of Ser-1177, under conditions of eNOS uncoupling (i.e., in absence of BH_4), increases the rate of superoxide anion generation instead of NO [37].

All the above studies demonstrate the intricate interplay existing between ROS and eNOS phosphorylation in Ser-1177 in relationship to BH_4 levels (which are reduced under elevated levels of oxidative stress or diabetes; see Figure 2). Therefore, unbalanced red/ox homeostasis, as found in diabetes, will determine an exacerbated production of superoxide anion instead of NO, leading to diabetic-associated vascular dysfunction.

2.2. Oxidative Stress Insulin Resistance and Diabetic Complications. Oxidative stress plays a pivotal role in the development of insulin resistance and in both micro- and macro-vascular diabetic complications [38].

Moreover, diabetic condition is also associated with an impairment of cellular autophagy, a process involved in the degradation of cellular components [39]. Autophagy and oxidative stress are strictly related since autophagy is also responsible for organelles degradation, such as mitochondria, which are the sites of ROS production. Autophagy impairment, in fact, causes an accumulation of dysfunctional mitochondria leading to increased ROS production. Therefore the autophagy impairment associated with diabetes plays a causal role in ROS increase [39]. In diabetic conditions, in fact, there is a mitochondrial superoxide overproduction in ECs of both large and small vessels, as well as in the myocardium. Superoxide production is responsible for the activation of five major pathways involved in the pathogenesis of diabetes complications: polyol pathway flux, increased formation of advanced glycation end products (AGEs), increased expression of the AGE receptor and its activating ligands, activation of protein kinase C isoforms, and overactivity of the hexosamine pathway [38]. Moreover, it directly inactivates two critical antiatherosclerotic enzymes, eNOS (see paragraph above) and prostacyclin synthase. Through these pathways, increased intracellular ROS provokes angiogenesis impairment in response to ischemia, activates a number of proinflammatory pathways, and causes long-lasting epigenetic changes that drive persistent expression of proinflammatory genes after glycemia being normalized (hyperglycemic memory), described below in Section 5. Atherosclerosis and cardiomyopathy in type 2 diabetes are caused in part by pathway-selective insulin resistance, which increases mitochondrial ROS production from free fatty acids and by inactivation of antiatherosclerotic enzymes by ROS [38].Indeed, overexpression of superoxide dismutase in transgenic diabetic mice prevents diabetic retinopathy, nephropathy, and cardiomyopathy [38].

An important role in the establishment of insulin resistance is played by the redox sensitive transcription factor NF-E2-related factor 2 (Nrf2) [40]. Among the different mechanisms used by the cell to counteract sustained oxidative stress, Nrf2 regulates antioxidant response element (ARE/EpRE-) mediated expression of detoxifying and antioxidant enzymes and of the cystine/glutamate transporter involved in glutathione biosynthesis [41]. Nrf2/ARE activity decrease causes oxidative stress increase and mitochondrial dysfunction in the vasculature, leading to endothelial dysfunction, insulin resistance, and abnormal angiogenesis associated with diabetes [42]. Moreover it has been shown that the suppression of Nrf2 activity by the MAPK extracellular-signal-regulated kinase (ERK) is linked to the oxidative stress-induced insulin resistance in mice [43]. Since ERK is known to be a negative regulator of glucose uptake and to be responsible of oxidative stress-induced insulin resistance, a strong link does exist between these phenomena. In addition, Nrf2 expression in the heart has been shown to be first upregulated and then downregulated in late stages of diabetes in the mouse. In this contest, Nrf2 activation has been demonstrated both to suppress oxidative stress-induced ERK activity and to reverse oxidative stress-induced insulin resistance. Finally, antioxidants such as N-acetyl cysteine (NAC) and metallothionein can prevent oxidative stress-induced ERK activation and Nrf2 downregulation [43].

These results demonstrate that Nrf2 plays a critical role in regulating insulin sensitivity in the heart but presumably also in other tissue districts; therefore, targeting Nrf2 might provide a novel therapeutic approach for the treatment of insulin resistance and diabetic cardiomyopathy [43].

2.3. NO Bioavailability and Inflammation. Acute inflammatory states are known to impair endothelium-dependent vasodilatation since inflammatory mediators, such as tumor necrosis factor-α (TNF-α), decrease eNOS expression in ECs [44]. The inflammatory process induces the activation of ECs that is characterized by adhesion molecule expression, eNOS decrease, and consequent loss of NO bioactivity, all mechanisms that are critical for the promotion of the atherogenic phenotype [45]. Diabetes is associated with a systemic inflammatory state that may impair endothelial function and contribute to atherosclerosis [46]. Indeed, in diabetic patients there is an increase of circulating levels of inflammatory markers, including C reactive protein, TNF-α, and intercellular adhesion molecule-1 [47–49]. Moreover, higher levels of inflammatory markers are a predictor of increased cardiovascular risk in diabetic patients [50] and, on the other hand, augmented levels of circulating inflammatory markers also relate to the incidence of diabetes mellitus [51].

The nuclear factor κB (NFκB) transcription factor, a key regulator of inflammatory genes transcription, is also implicated in endothelial activation and is linked to the

pathogenesis of insulin resistance [52]. Inflammatory mediators, such as inflammatory cytokines, free fatty acids, and the AGE receptor, activate NFκB. They all induce the phosphorylation of the inhibitory subunit IκB by the IκB kinase (IKK-β), which allows the translocation of the regulatory subunits p50 and p65 to the nucleus, where they promote the expression of inflammatory genes. Indeed, overexpression of IKK-B or TNF-α in skeletal muscles causes insulin resistance [53]. Accordingly, *in vitro* and *in vivo* studies confirmed the existence of a link between insulin resistance and NFκB activation, inflammation, and NO bioactivity impairment [54]. As a further confirmation, genetic suppression of IKK-β or pharmacological inhibition of IKK-β with a class of drugs named salicylates prevents insulin resistance. Accordingly, a large randomized trial was conducted in type 2 diabetes patients (TINSAL-T2D) testing an anti-inflammatory agent belonging to salicylate group, named salsalate (2-(2-hydroxybenzoyl) oxybenzoic acid). It was found that salsalate lowers hemoglobin A (1c) levels and improves glycemic control, showing the importance of chronic inflammation as pathogenetic mechanism of diabetes.

3. p66^{Shc} and Intracellular Oxidative Stress

In the oxidative stress-mediated responses, Shc proteins, intracellular adaptors regulating a variety of cellular functions, assume a major role [55]. There are three Shc mammalian genes: ShcA, ShcB, and ShcC. In mammals, ShcA is ubiquitously expressed, while ShcB and ShcC expression are limited to neuronal cells [56, 57].

The ShcA adaptor protein has three isoforms of 46, 52, and 66 kDa (p46^{Shc}, p52^{Shc}, and p66^{Shc}, resp.) all generated from the same transcript, either through RNA splicing or alternative translational initiation [58, 59]. These three isoforms all share a common structure made of the CH_2–PTB–CH_1–SH_2 modular domains [60]; the C terminal SH_2 domain is necessary to bind to phosphotyrosine containing sequences; the PTB domain is a second domain capable of interacting with phosphorylated tyrosine residues independently; in particular, the p46^{Shc} isoform lacks the first 46 amino acids within the PTB domain. The CH_1 domain lays between the PTB and SH_2 domains and contains tyrosine residues that, upon phosphorylation, activate a specific signaling cascade. Finally, p66^{Shc} only has an additional domain at the N terminus, the CH_2 domain. Of relevance, this domain contains a serine residue at position 36 (Ser-36) that is phosphorylated in response to several stress stimuli, including UV irradiation and H_2O_2 [61].

p52^{Shc} and p46^{Shc} proteins are inductors of the Ras signaling pathway [62–64]. In particular, p52^{Shc} is efficiently phosphorylated by the insulin receptor, causing the activation of the MAPK pathway that leads to cellular growth and proliferationviathe small GTPase Ras [22]. Interestingly, this pathway is not inhibited in diabetes mellitus and insulin resistance; conversely, the insulin-mediated activation of eNOS viaPI3 kinase/AKT1 is inhibited, limiting its vasodilator and prosurvival function and therefore promoting vascular disease [24].

The p66^{Shc} isoform function is not limited to signal transduction, since it is a redox enzyme implicated in mitochondrial ROS generation and translation of oxidative signals [55].

Under physiological conditions, the phosphorylation of Tyr residues of p66^{Shc} by growth factors mediates the signal transduction to the nucleus, inhibiting the Ras signaling pathway, while phosphorylation of the Ser-36 site seems to be crucial for oxidative stress response [65–67].

p66^{Shc} modulates ROS production by using three mechanisms restricted in the nucleus, the plasma membrane, and the mitochondria, respectively. The nuclear mechanism involves p66^{Shc} mediated by forkhead box sub-group O (FOXO) transcription factors inhibition, leading to the decreased expression of ROS-scavenging enzymes CAT and manganese superoxide dismutase (MnSOD) [68]. At the plasma membrane, p66^{Shc} promotes RAC1 activation and triggers NADPH membrane oxidase-ROS production. A positive feedback loop between RAC1 and p66^{Shc} exists: RAC1, in fact, induces the phosphorylation-dependent increase of p66^{Shc} stability [69]. Finally, p66^{Shc} acts in the mitochondrial intermembrane space. In response to oxidative stress, p66^{Shc} is serine phosphorylated by protein kinase CβII (PKCβII) and isomerized by the peptidylprolyl cis/trans isomerase PIN-1 [70, 71]; this isomerization allows the dephosphorylation of Ser-36 residue by the serine threonine phosphatase PP2A, inducing the translocation from the cytosol to the mitochondrial intermembrane space, through the TIM/TOM mitochondrial import machinery. In the mitochondrial intermembrane space, p66^{Shc} binds to cytochrome c, acting as an oxidoreductase and generating ROS. These ROS, in turn, activate the permeability transition pore, triggering organelle dysfunction, massive release of mitochondrial apoptotic factors, and ROS and eventually inducing cell apoptosis [71] (Figure 3).

Indeed, increased p66^{Shc} content in the mitochondria correlates with alteration of mitochondrial structure, decrease of mitochondrial calcium uptake, and enhanced mitochondrial ROS production, triggering the mitochondrial route of apoptosis [70]. It is worth noting that the mitochondrial H_2O_2 production induced by p66^{Shc} further increases intracellular H_2O_2 levels, maintaining or increasing PKCβII activation in a positive control loop [72, 73].

In keeping with its proapoptotic function, p66^{Shc} can be also phosphorylated by apoptosis signal-regulating kinase 1 (ASK1) [74] and p38 MAPK [75]. Indeed, p38 MAPK is target of several factors inducing ROS generation, including osmotic and thermic shock, inflammatory cytokines, lipopolysaccharides, ultraviolet light, interleukin 1, and H_2O_2 [75].

In agreement with these data, increased resistance to oxidative stress has been observed in p66^{Shc} knockout mice (p66$^{Shc-/-}$) [61] and characterized by reduced oxidative stress-induced apoptosis, prolonged lifespan, reduced production of intracellular oxidants, and increased resistance to oxidative stress-induced apoptosis, which is restored by p66^{Shc} overexpression [61]. Consistently, p66$^{Shc-/-}$ mice show

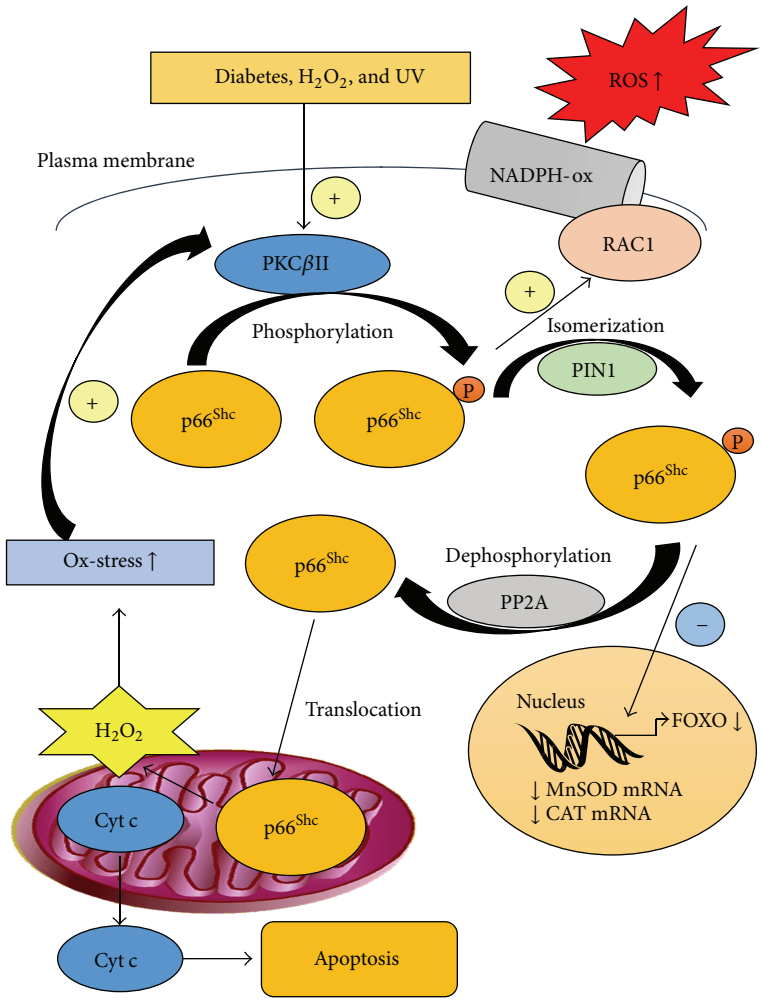

Figure 3: $p66^{Shc}$ role in intracellular ROS production. $p66^{Shc}$ modulates ROS production by using three mechanisms. (1) The nuclear mechanism involves $p66^{Shc}$ mediated FOXO transcription factors inhibition, leading to decreased expression of ROS-scavenging enzymes such as catalase (CAT) and manganese superoxide dismutase (MnSOD). (2) At the plasma membrane, $p66^{Shc}$ promotes RAC1 activation and triggers NADPH membrane oxidase-ROS production. (3) $p66^{Shc}$ acts in the mitochondrial intermembrane space (IMS). In response to oxidative stress, $p66^{Shc}$ is serine phosphorylated by PKCβII and isomerized by the peptidylprolyl cis/trans isomerase PIN1. This isomerization allows the dephosphorylation of Ser-36 residue by the serine threonine phosphatase PP2A, inducing the translocation from the cytosol to the IMS. In the IMS, $p66^{Shc}$ binds to cytochrome c (Cyt c), generating ROS. These ROS activate the release of mitochondrial apoptotic factors, eventually inducing apoptosis.

reduced levels of systemic (isoprostane) and tissue (nitrotyrosine, 8-oxo-dG) oxidative stress markers [68, 76, 77] and enhanced resistance to oxidative stress induced by hypercholesterolemia [78], angiotensin II [79], carbon tetrachloride, and ethanol [80]. Indeed, our group demonstrated that $p66^{Shc}$ deletion increased both skeletal muscle and EC resistance to ischemia [81]. Intriguingly, we also found that $p66^{Shc}$ not only inhibited cell survival but also differentiation of skeletal muscle progenitors and skeletal muscle regeneration after hindlimb ischemia [82].

These observations seem to be of clinical relevance. Indeed, $p66^{Shc}$ mRNAs is increased in peripheral blood monocytes of patients with acute coronary syndrome, but not in those who display stable coronary artery disease [83]. In addition, plasma levels of malondialdehyde, an established marker of lipid peroxidation and thus of oxidative stress, correlate positively with $p66^{Shc}$ expression.

3.1. $p66^{Shc}$, ROS and Diabetes Mellitus. Different reports unravel a major role of $p66^{Shc}$ also in response to hyperglycemia (HG) and diabetes conditions that both upregulate oxidative stress.

There are several studies exploring the interactions between hyperglycemia-associated ROS production and $p66^{Shc}$ in diabetes animal models. Indeed, streptozotocin (STZ) treated mice, a model of type 1 diabetes, show higher expression of $p66^{Shc}$ compared to nondiabetic mice. In addition, STZ treated $p66^{Shc-/-}$ and wild type (wt) mice show a similar increase in blood glucose but significant differences

with respect to endothelial dysfunction and oxidative stress production [84]. Peroxynitrites are formed after the reaction between free radicals, such as superoxide and NO, decreasing NO availability and enhancing cellular oxidative stress and eventually leading to endothelial dysfunction [85]. In accordance with the decreased oxidative stress levels observed upon diabetes induction, $p66^{Shc-/-}$ mice also display reduced peroxynitrite production, and thus contributing to blood vessel relaxation [84].

Increased glucose levels impact directly on $p66^{Shc}$. Indeed, HG-mediated ROS production induces phosphorylation of the Ser-36 residue of $p66^{Shc}$, leading to the collapse of mitochondrial transmembrane potential [86].

A well-known effect of HG is the formation and accumulation of AGEs, which can further amplify oxidative damage by increasing oxidative stress in cells [87, 88]. Ser-36 $p66^{Shc}$ phosphorylation mediated through AGE-induced ROS production has been shown to be responsible for FOXO3A inhibition [89].

Additionally,HG-mediated ROS overproduction also activates AKT1/PKB kinase, which phosphorylates and inactivates FOXO3A protein, inducing oxidative stress and depressing the survival phenotype [90]. This is of particular importance for diabetes-associated redox imbalance, since FOXO3A transcription factor is responsible for SOD2 and catalase ROS scavenger synthesis [91–93]. $p66^{Shc}$-AKT interplay also affects NO production: $p66^{Shc}$ silencing leads to activation of Ras and AKT kinase, with a corresponding increase in phosphorylation of eNOS at Ser-1177. Accordingly, in rat aortic rings, knockdown of $p66^{Shc}$ suppresses the vasoconstrictor responses enhancing vasodilatation [94].

Another signaling pathway altered in HG involves NFκB and NOX4 (NADPH oxidase 4),that are elevated in $p66^{Shc}$ wt but not in knockout animals [95].

Finally, $p66^{Shc}$ is essential also for glucose uptake in skeletal muscle cells. In fact, $p66^{Shc}$ protein regulates MAPK activity and the actin cytoskeleton turnover [96], which are both required for normal glucose transport regulation. Loss of $p66^{Shc}$ in rat myoblasts activates MAPK activity, leading to altered cell cytoskeleton and resulting in strong increase in basal glucose transport [97]. Moreover glucose transporter proteins GLUT1 and GLUT3 are induced too. On the other hand, in rat myoblasts overexpressing $p66^{Shc}$, basal glucose uptake rate is significantly reduced and the cellular levels of glucose transporters GLUT1 and GLUT3 are decreased. Thus, $p66^{Shc}$ may represent an effector of glucose transport in skeletal muscle cells and confirm to play an important role in the adaptive responses to environmental factors [97].

Again, these observations appear to be of clinical relevance. Indeed, $p66^{Shc}$ mRNA level is significantly increased in mononuclear blood cells from type 2 diabetic patients compared to healthy controls and it correlates positively with total plasma isoprostanes, well-known markers of oxidative stress [98].

3.1.1. $p66^{Shc}$ and Endothelial Progenitor Cells (EPCs) Function in Diabetes Mellitus. EPC levels in diabetic patients are significantly reduced compared with control subjects. EPCs derived from diabetic patients, in fact, produce excessive ROS and show impaired migratory capacity [99]. eNOS uncoupling explains, at least in part, the reduced levels and impaired function of EPCs observed in diabetes contributing to the pathogenesis of vascular disease [99].

Accordingly, mouse bone marrow derived c-kit$^+$ cells of $p66^{Shc-/-}$ mice are resistant to both apoptosis and oxidative stress induced by high glucose [100]. Upon oxidative stress, the bioavailability of NO is reduced, and consequently endothelial function and differentiation are impaired. HG resistance conferred by $p66^{Shc}$ deletion is dependent on the activity of NOS and, accordingly, a NO donor is sufficient to rescue bone marrow-derived EPCs deficit induced by HG [100]. In line with *in vitro* data, the knockout of $p66^{Shc}$ prevents the diabetic impairment of capillary network formation in a mouse model of angiogenesis [100], strongly indicating that $p66^{Shc}$ represents a promising therapeutic target for the prevention, the development, and the progression of diabetic vasculopathy.

3.2. $p66^{Shc}$ Role in Hypercholesterolemia. Hypercholesterolemia increases ROS and reactive nitrogen species production, resulting in oxidation and peroxidation of lipids, proteins, and lipoproteins [101–104]. In $p66^{Shc-/-}$ mice chronically fed with a high-fat diet, the levels of oxidized low-density lipoprotein (LDLs) and of isoprostanes, produced from polyunsaturated fatty acids through radical-catalyzed mechanisms, are reduced as well as the formation of intimal macrophage-derived foam cells in the arterial wall. Thus, loss of $p66^{Shc}$ expression protects against oxidative stress and early lesion formation [78]. Furthermore, in $p66^{Shc-/-}$ mice, low atherogenesis and reduced oxidative stress are coupled with reduced apoptosis in aortic lesions [78].

In an interesting study, the relationships among lipids, oxidative stress, and $p66^{Shc}$ were investigated in peripheral white blood cells and in subcutaneous adipose specimens of patients displaying either high or low LDL plasma levels [105]. It was reported that $p66^{Shc}$ mRNA levels in WBC and in adipose tissue were directly related to LDL expression, and multiple regression analysis showed that LDL plasma levels were the only variable affecting $p66^{Shc}$ mRNA expression [105].

3.3. $p66^{Shc}$ Role in Aging. Aging is an independent risk factor for cardiovascular diseases and senescent vascular cells are present in human atherosclerotic tissues [106], suggesting that vascular cell senescence could be linked to the pathophysiology of age-related vascular diseases. Accordingly, vascular cell senescence has been also shown in diabetic vasculopathy [107, 108]. Indeed, HG-induced endothelial senescence leads to vascular inflammation and thrombosis, exacerbating the diabetic-associated cardiovascular events.

Calorie restriction in mammals decreases the incidence of age-associated disorders including cardiovascular diseases [109]. "Silent mating type information regulation 2 homolog" (sirtuin 1 or SIRT1) is NAD$^+$ -dependent class III

Table 1: $p66^{Shc}$ knockout mice phenotypes.

Disease	Phenotype	$p66^{Shc+/+}$	$p66^{Shc-/-}$
Diabetes	Peroxynitrite production	+++	+
	Lipid peroxidation	+++	+
	$p66^{Shc}$ expression	++	−
Aging	NO availability	+	+++
	O_2 production	+++	+
	Protein nitration	+++	+
	iNOS expression	+++	+
Hypercholesterolemia	Aortic lesions area	+++	+
	Plasmatic isoprostanes	+++	+
	Lipid peroxidation	+++	+
	Vascular apoptosis	+++	+

+++: high expression or production; ++: medium expression or production; +: moderate expression or production; −: no expression.

histone deacetylase (HDAC) found up-regulated under caloric restriction and extending the lifespan of many organisms [110]. A SIRT1 upregulation and/or activation is associated with EC functional preservation; whereas excessive ROS or aging decrease SIRT1 expression, leading to endothelial dysfunction [111]. In fact, SIRT1 activation in ECs ameliorates oxidative stress response, prevents endothelial senescence, and promotes eNOS-derived NO bioavailability and mitochondrial biogenesis [112–114].

SIRT1 has been shown to target p53, FOXO3, and eNOS for deacetylation, negatively regulating oxidative stress [114–116]. Recently, it has been demonstrated that the endothelium-specific overexpression of SIRT1 is able to inhibit the HG-induced upregulation of the senescence-associated markers, such as the CDK inhibitor $p21^{Waf1/Cip1/Sdi1}$ (p21), p53, and the plasminogen activator inhibitor-1 (PAI-1) [117]. Furthermore, SIRT1-transgenic diabetic mice exhibited decreased expression of $p66^{Shc}$ and increased expression of the scavenging enzyme MnSOD [117]. Indeed, SIRT1 has been shown to repress $p66^{Shc}$ transcription at the chromatin level: SIRT1 overexpression decreased acetylated histone H3 binding to the $p66^{Shc}$ promoter region; whereas inhibition of SIRT1 increased acetylated histone H3 binding to the same region. Therefore, the decreased levels of $p66^{Shc}$ attributable to SIRT1 could be the result of a direct inhibitory role of SIRT1 on $p66^{Shc}$ expression through epigenetic chromatin modifications [112].

Overall, these data suggest that the protective role of SIRT1 against hyperglycemia-induced vascular cell senescence is mediated, at least in part, through the reduction of oxidative stress through a cross-talk with $p66^{Shc}$.

The above described role of $p66^{Shc}$ in endothelial dysfunction associated with different disease conditions is summarized in Table 1.

4. MicroRNAs Oxidative Stress and Diabetes

MicroRNAs (miRNAs) are small noncoding RNAs that regulate stability and translational inhibition of target messenger RNAs (mRNAs). miRNAs are involved in most biological processes, including proliferation, differentiation, development, migration, and apoptosis [11, 12]. miRNA dysregulation has been observed in the development of different diseases, including diabetes mellitus [13–15]. Specifically, several miRNAs modulated by oxidative stress have been demonstrated to be dysregulated in diabetes and to cause vasculopathy (Table 2).

Among them, miR-200 family members have been shown to play a causative role in the establishment of vascular diabetic inflammatory phenotype [118]. This miRNA family consists of five members, miR-200c, miR-141, miR-200a, miR-200b, and miR-429 and it has been widely studied for its role in the epithelial to mesenchymal transition of tumor cells [119].

We found that miR-200 family is induced upon H_2O_2 treatment in ECs and in particular one of its members, miR-200c, is responsible for the induction of apoptosis and senescence [120]. This is likely physiologically relevant since miR-200 family induction is also observed in a mouse model of hindlimb ischemia with an oxidative stress mediated mechanism [81]. Indeed, miR-200c and miR-200b upregulation is markedly inhibited in ischemic $p66^{Shc-/-}$ mice, which display lower levels of oxidative stress in basal conditions [10] and after ischemia [81], supporting a role of ROS in miR-200 family induction [120].

An upregulation of miR-200 family members has been also reported in VSMCs of diabetic mice (db/db) compared to control db/+ mice [118]. In particular, the authors found that miR-200b, miR-200c, and miR-429 expression levels were increased in VSMCs of diabetic mice, while the protein levels of their common target ZEB1 were decreased. Overexpression of miR-200 mimics the downregulated transcriptional repressor ZEB1, leading to the transcription of the inflammatory genes cyclooxygenase-2 (COX-2) and monocyte chemoattractant protein-1 (MCP-1) that in turn promote monocyte binding to VSMCs, eliciting a proinflammatory response. In accordance with these results, ZEB1 occupancy of inflammatory gene promoters was reduced in db/db VSMCs [118].

Notably, miR-200 family is also induced by NO [121]. NO treatment and miR-200-family overexpression, or ZEB2 knockdown, all elicit the expression of mesendoderm and early cardiovascular precursor markers, including fetal liver kinase 1 (Flk1) and chemokine receptor type 4 (CXCR4), inducing mouse embryonic stem (ES) cells differentiation towards the mesoendoderm and cardiovascular lineage [121].

Another miRNA that is found significantly upregulated in VSMCs of diabetic mice (db/db) compared to control mice db/+ is miR-125b [122] that is also induced by oxidative stress in human keratinocytes HaCaT exposed to H2O2 [123]. miR-125b protein target is the histone H3 lysine-9 methyltransferase Suv39 h1. In diabetic VSMCs, there is a decreased promoter occupancy of Suv39 h1 in inflammatory genes and, consequently, of the associated repressive epigenetic

Table 2: Modulated miRNAs in diabetic endothelial dysfunction and oxidative stress.

ROS source/disease	miRNAs	Tissue/organ	Source	Target	Functions	References
	Upregulated					
H_2O_2	miR-200c, miR-141, miR-200a, miR-200b, and miR-429	ECs, myoblasts	Human	ZEB1	Apoptosis, senescence	[120]
Diabetes	miR-200c, miR-200b, and miR-429	VSMCs	Mouse	ZEB1	Inflammation	[118]
NO	miR-200c, miR-200a, miR-200b, and miR-429	mES	Mouse	ZEB2	Mesendoderm and cardiovascular differentiation	[121]
Hypoxia	miR-210	ECs	Human	EFNA3	Angiogenesis	[125]
Diabetes	miR-210	Failing heart	Human			[127]
Diabetes	miR-125	VSMCs	Mouse	Suv39h1	Inflammation	[122]
	Downregulated					
Diabetes	miR-27b	BMACs	Human/mouse	Sema6A, $p66^{shc}$, and TSP-1	ROS production; angiogenesis impairment	[124]

BMACs: bone marrow-derived angiogenic cells, ECs: endothelial cells, EFNA3: Ephrin A3, H_2O_2: hydrogen peroxide, mES: mouse embryonic stem, NO: nitric oxide, $p66^{Shc}$: p66 isoform of ShcA protein, ROS: reactive oxygen species, Sema6A: semaphorin 6A, Suv39h1: suppressor of variegation 3-9 homolog 1 (Drosophila), TSP-1: thrombospondin-1, VSMCs: vascular smooth muscle cells, ZEB1: zinc finger E-box binding homeobox 1, and ZEB2: zinc finger E-box binding homeobox 2.

mark histone H3 lysine-9 trimethylation (H3K9me3) [122]. This study supports the idea that epigenetic mechanisms implicated in the upregulation of inflammatory genes in ECs and VSMCs under diabetic conditions, at least in part, pass through oxidative stress-dependent modification of miRNA expression (see section below).

Recently, it has been shown that decreased levels of miR-27b are present in bone marrow-derived angiogenic cells (BMACs) from both type 2 diabetes mellitus patients and type 2 diabetic db/db mice [124]. miR-27b under normoglycemic condition protects BMAC angiogenesis, represses mitochondrial reactive oxygen species, and improves wound healing by targeting the antiangiogenic molecules semaphorin 6A (Sema6A), $p66^{Shc}$, and thrombospondin-1 (TSP-1), respectively [124]. In contrast, in diabetes mellitus, miR-27b expression is decreased, which harms BMAC angiogenesis and increases mitochondrial ROS production [124]. Overexpression of miR-27b, in fact, rescues BMAC functions and improves BMAC therapy on diabetic wounds, accelerating wound closure and increasing wound perfusion. These data indicate that miR-27b gene therapy enhances the efficacy of diabetic angiogenic cells for wound angiogenesis and wound repair in diabetic subjects [124].

Another miRNA that plays a major role in EC function is miR-210 [125]. In particular, its upregulation stimulates EC angiogenesis, at least in part, through the downmodulation of Ephrin A3 (EFNA3) [125]. Moreover, miR-210 is involved in mitochondrial ROS production targeting many mitochondrial components [3] and miR-210 blockade decreases EC survival upon ischemia with an oxidative stress mediated mechanism [126]. Interestingly, miR-210 levels are also induced in the failing heart of postischemic type 2 diabetic patients [127]. Thus, while miR-210 levels have not been measured in cardiac endothelium specifically, dysregulation of this miRNA is likely to play a role in diabetic EC dysfunction.

5. Epigenetic Modulations Induced by ROS in Diabetes Mellitus

As previously described, epigenetic modulations elicited under diabetic conditions have been proven to play a pivotal role in the progression of the disease and, importantly, in hyperglycemic memory. The latter likely underpins the failure of intensive glucose control in the improvement of cardiovascular outcomes in diabetic patients [9].

In vitro studies demonstrate that epigenetic mechanisms modulate glucose-induced gene expression of the subunit p65 of NFκB. This phenomenon has been assigned to the activation of SET7/9 histone methyltransferase which methylates lysine 4 on histone H3 and promotes gene transcription [128]. The overall process leads to NFκB activation and to the expression of proinflammatory molecules such as VCAM-1 and MCP-1.

The emerging concept is that epigenetic modifications are at the basis of high glucose-dependent modifications and may be involved in the onset of hyperglycemic memory. Although this observation still awaits confirmation in humans, it is certainly intriguing and worth of further investigations. A possible problem could be that not all the cells in the body may be equally sensitive to high glucose, resulting in a persistent modification of gene expression and/or cell function. Our knowledge in this direction is still limited and further analyses are required to understand the molecular basis of hyperglycemic/epigenetic memory as well as the

potential interventions we may design to correct the problem [129]. Intriguingly, the same proinflammatory genes are induced in models of diabetes-associated atherosclerosis, suggesting that similar mechanistic processes may underlie different physiopathological outcomes associated with the same metabolic alteration [130, 131].

Evidence is emerging that ROS could be key mediators underpinning glucose mediated epigenetic modulations; in particular, mitochondrial ROS have been shown to be implicated in the epigenetic changes that induces NFκB activation [129, 132]. Recently, a study shows that. in ECs exposed to high glucose and in aortas of diabetic mice, activation of $p66^{Shc}$ by PKCβII continues after returning to normoglycemia [133]. This persistent $p66^{Shc}$ upregulation and mitochondrial translocation is associated with continued ROS production, apoptosis, and reduced NO bioavailability. In particular, $p66^{Shc}$ gene is epigenetically regulated by its promoter CpG hypomethylation and by GCN 5-induced histone 3 acetylation, causing $p66^{Shc}$ overexpression [133]. Accordingly, also other oxidative stress inducing stimuli seem to act with a similar mechanism: cholesterol upregulates EC $p66^{Shc}$ expression via hypomethylation of two CpG dinucleotides and acetylation of histone 3 in its promoter [134].

Furthermore, $p66^{Shc}$-derived ROS production induces PKCβII upregulation that, in turn, phosphorylates and inhibits eNOS, leading to a damaging vicious cycle even after restoration of normoglycemia. *In vivo* and *in vitro* gene silencing of $p66^{Shc}$ after glucose normalization restores endothelium-dependent vasorelaxation and, decreasing ROS production, inhibits apoptosis [133].

The above described studies underline the importance of the relationship occurring between $p66^{Shc}$, ROS production, eNOS activity, and inflammation that all concur to the establishment of the hyperglycemic memory and of vascular diseases associated with diabetes.

6. Clinical Use of Antioxidants to Improve Endothelial Function

Given the fundamental role of oxidative stress in the onset of diabetes and diabetic complications, several attempts to target redox imbalance pharmacologically have been conducted [135].

A number of interventional trials were conducted between 1996 and 2002, testing vitamin E, β-carotene, and vitamin C, alone or in combination, and at different dosages. Although some studies showed a benefit of vitamin E administration in the secondary prevention of cardiovascular disease [136] and of vitamin E + C supplementation in slowing carotid intima-media thickening in hypercholesterolaemic patients [137], clinical trials gave heterogeneous outcomes. Therefore, in 2004, the American Heart Association Committee for Nutrition, Physical Activity, and Metabolism discouraged the use of antioxidant for cardiovascular disease prevention [138].

The neutral effect of vitamin E + C administration was also confirmed by the results of the 2008 Physicians Health Study that enrolled more than 14000 middle-aged male with low prevalent cardiovascular disease and followed them up for about 10 years [139]. Importantly, questions have been raised about the safety of antioxidant vitamins, since an increased overall mortality associated with β-carotene, vitamin E, and vitamin A supplementation was reported in some of these trials, possibly due to increased cancer mortality [140]. In conclusion, these interventional trials did not confirm that pharmacological correction of the redox status with antioxidant vitamins could be used as a safe and effective therapeutic strategy.

However, another group of antioxidant compounds, polyphenols, comprising about 8000 different molecules, among which flavonoids are the most studied, holds good promise. Polyphenols are abundant in vegetables and particularly in products such as wine, chocolate, and tea. The antioxidant capacity of these compounds is attributable both to the inhibition of enzymatic sources of ROS and to the stimulation of antioxidant mechanisms [141]. Indeed, a meta-analysis of 113 studies testing different food, beverages, or extracts supplementations demonstrated that polyphenols improve endothelial function, both in healthy subjects and in patients with cardiovascular risk factors, reducing blood pressure [142].

One of the most studied polyphenols is resveratrol that is contained in red grapes as well as in other fruits. Animal studies demonstrated that resveratrol displays a strong antidiabetic effect, decreasing blood glucose in hyperglycemic rodents [143]. This effect seems to result from increased intracellular transport of glucose. Moreover, resveratrol was also demonstrated to have effects that may contribute to the protection of β-cells in diabetes. Indeed, in rat pancreatic islets experiments, resveratrol was shown to reduce insulin secretion [143] and this event was confirmed in rats with hyperinsulinemia, in which resveratrol decreased blood insulin levels [144]. Moreover, resveratrol inhibited cytokine action and attenuated the oxidative damage of the pancreatic tissue [145]. Additionally, studies in animal models of insulin resistance indicate that resveratrol increases insulin function (reviewed in [144]). The improvement of insulin action mechanism is complex and involves reduced adiposity and changes in gene expression and in the activities of different enzymes. Finally, resveratrol has been demonstrated to increase Nrf2 activity, supporting its antioxidant effect [146] and to be a potent inducer of SIRT1, a key molecule that regulates energy homeostasis, mitochondrial biogenesis, and insulin sensitivity, as above described [147].

In spite of the encouraging preclinical data, clinical intervention studies performed so far show conflicting effects of resveratrol between trials (reviewed in [148]). Some trials revealed an insulin sensitivity increase and glucose control, whereas others did not report positive effects [148]. Factors that may influence the outcome of the trials are resveratrol doses and the timing of consumption as well as the metabolic status of the subject.

Another approach aimed at modulating oxidative stress in humans takes advantage of drugs directed primarily to other targets. Angiotensin-receptor blockers and ACE inhibitors have been found to exert nonhemodynamic

beneficial effects on endothelial function by inhibiting NOX activity, reducing subunits expression and vascular oxidative stress [149]. Similarly, statins reduce NOX-mediated ROS production and activate NOS function [150]. After a short time of treatment with statins, an improvement in the endothelial function of the forearm vasculature was demonstrated, prior to any lipid-reducing effect [151]. In summary, the beneficial effects obtained by a therapy with ACE inhibitors and/or statins seem to be, at least in part, mediated by properties that are independent of the hemodynamic or cholesterol-lowering effects of these drugs.

Another approach to prevent ROS generation is inhibiting the enzyme system xanthine-oxidase, a well-known endothelial and cardiac source of superoxide [152]. Indeed, in one trial, the inhibition of xanthine-oxidase with allopurinol was shown to improve endothelial dysfunction in patients with type 2 diabetes with mild hypertension [153].

Several explanations can be envisioned to explain the largely conflicting results obtained so far in interventional trials testing antioxidant strategies. It must be underlined that ROS play a fundamental role as second messengers in cell physiology and that in low concentrations some ROS, particularly H_2O_2, are very important for cell growth and angiogenesis; moreover, a crucial role of ROS has also been shown in protective mechanisms such as preconditioning [154]. Therefore, the administration of antioxidants that is based on the concept that these molecules only have damaging effects should be reconsidered since, in certain settings, ROS quenching might have deleterious implications that counterbalance the positive ones. Methodological issues also need to be carefully considered: for instance, dosage, treatment duration, choice of outcome measures, populations under study, and concomitant therapy [155].

Collectively, these considerations provide a sufficient explanation for the failure of antioxidants tested so far. Evidence indicates that we need more hypothesis-driven and rigorous clinical trial designs, guided by a better understanding of the complex physiopathological role of ROS. Future research will have to develop newer antioxidant molecules, more specific, with a better pharmacodynamic profile or ancillary effects or impacting systemic and tissue oxidative stress through different mechanisms.

7. Conclusions

We described in this review how oxidative stress, modulating $p66^{Shc}$-NO pathway, impinges in many crucial aspects of diabetic endothelial dysfunction, representing a promising therapeutic objective. So far, however, it is still unclear which pathogenetic mechanism should be targeted and the class of drugs that may be useful. In our vision, a more accurate knowledge of the fundamental disease mechanisms is the only way to the identification of a therapeutic strategy targeting oxidative stress.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] J. E. Deanfield, J. P. Halcox, and T. J. Rabelink, "Endothelial function and dysfunction: testing and clinical relevance," *Circulation*, vol. 115, no. 10, pp. 1285–1295, 2007.

[2] C. Napoli and L. J. Ignarro, "Nitric oxide and pathogenic mechanisms involved in the development of vascular diseases," *Archives of Pharmacal Research*, vol. 32, no. 8, pp. 1103–1108, 2009.

[3] A. Magenta, S. Greco, C. Gaetano et al., "Oxidative stress and microRNAs in vascular diseases," *International Journal of Molecular Sciences*, vol. 14, no. 9, pp. 17319–17346, 2013.

[4] D. D. Gutterman, H. Miura, and Y. Liu, "Redox modulation of vascular tone: focus of potassium channel mechanisms of dilation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 4, pp. 671–678, 2005.

[5] H. Miki and Y. Funato, "Regulation of intracellular signalling through cysteine oxidation by reactive oxygen species," *Journal of Biochemistry*, vol. 151, no. 3, pp. 255–261, 2012.

[6] Y. Yang, A. Huang, G. Kaley, and D. Sun, "eNOS uncoupling and endothelial dysfunction in aged vessels," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 297, no. 5, pp. H1829–H1836, 2009.

[7] Y. Higashi, K. Noma, M. Yoshizumi, and Y. Kihara, "Endothelial function and oxidative stress in cardiovascular diseases," *Circulation Journal*, vol. 73, no. 3, pp. 411–418, 2009.

[8] P. D. Ray, B. Huang, and Y. Tsuji, "Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling," *Cellular Signalling*, vol. 24, no. 5, pp. 981–990, 2012.

[9] M. E. Cooper and A. El-Osta, "Epigenetics: mechanisms and implications for diabetic complications," *Circulation Research*, vol. 107, no. 12, pp. 1403–1413, 2010.

[10] M. Giorgio, M. Trinei, E. Migliaccio, and P. G. Pelicci, "Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals?" *Nature Reviews Molecular Cell Biology*, vol. 8, no. 9, pp. 722–728, 2007.

[11] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[12] E. Huntzinger and E. Izaurralde, "Gene silencing by microRNAs: contributions of translational repression and mRNA decay," *Nature Reviews Genetics*, vol. 12, no. 2, pp. 99–110, 2011.

[13] C. Guay, E. Roggli, V. Nesca, C. Jacovetti, and R. Regazzi, "Diabetes mellitus, a microRNA-related disease?" *Translational Research*, vol. 157, no. 4, pp. 253–264, 2011.

[14] A. K. Pandey, P. Agarwal, K. Kaur, and M. Datta, "MicroRNAs in diabetes: tiny players in big disease," *Cellular Physiology and Biochemistry*, vol. 23, no. 4–6, pp. 221–232, 2009.

[15] M. van de Bunt, K. J. Gaulton, L. Parts et al., "The miRNA profile of human pancreatic islets and beta-cells and relationship to type 2 diabetes pathogenesis," *PLoS ONE*, vol. 8, no. 1, Article ID e55272, 2013.

[16] R. Muniyappa and M. J. Quon, "Insulin action and insulin resistance in vascular endothelium," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 10, no. 4, pp. 523–530, 2007.

[17] J. A. Ware, D. D. Heistad, J. Flier, E. Salzman, and P. Weller, "Platelet-endothelium interactions," *The New England Journal of Medicine*, vol. 328, no. 9, pp. 628–635, 1993.

[18] M. A. Vincent, M. Montagnani, and M. J. Quon, "Molecular and physiologic actions of insulin related to production of nitric oxide in vascular endothelium," *Current Diabetes Reports*, vol. 3, no. 4, pp. 279–288, 2003.

[19] M. Federici, A. Pandolfi, E. A. de Filippis et al., "G972R IRS-1 variant impairs insulin regulation of endothelial nitric oxide synthase in cultured human endothelial cells," *Circulation*, vol. 109, no. 3, pp. 399–405, 2004.

[20] E. R. Duncan, P. A. Crossey, S. Walker et al., "Effect of endothelium-specific insulin resistance on endothelial function in vivo," *Diabetes*, vol. 57, no. 12, pp. 3307–3314, 2008.

[21] Z. Y. Jiang, Y. Lin, A. Clemont et al., "Characterization of selective resistance to insulin signaling in the vasculature of obese Zucker (fa/fa) rats," *The Journal of Clinical Investigation*, vol. 104, no. 4, pp. 447–457, 1999.

[22] C. Nacci, M. Tarquinio, and M. Montagnani, "Molecular and clinical aspects of endothelial dysfunction in diabetes," *Internal and Emergency Medicine*, vol. 4, no. 2, pp. 107–116, 2009.

[23] S. B. Wheatcroft, I. L. Williams, A. M. Shah, and M. T. Kearney, "Pathophysiological implications of insulin resistance on vascular endothelial function," *Diabetic Medicine*, vol. 20, no. 4, pp. 255–268, 2003.

[24] M. Montagnani, I. Golovchenko, I. Kim et al., "Inhibition of phosphatidylinositol 3-kinase enhances mitogenic actions of insulin in endothelial cells," *The Journal of Biological Chemistry*, vol. 277, no. 3, pp. 1794–1799, 2002.

[25] R. Stocker and J. F. Keaney Jr., "Role of oxidative modifications in atherosclerosis," *Physiological Reviews*, vol. 84, no. 4, pp. 1381–1478, 2004.

[26] A. San Martín, P. Du, A. Dikalova et al., "Reactive oxygen species-selective regulation of aortic inflammatory gene expression in type 2 diabetes," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 5, pp. H2073–H2082, 2007.

[27] L. Gao and G. E. Mann, "Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling," *Cardiovascular Research*, vol. 82, no. 1, pp. 9–20, 2009.

[28] C. E. Tabit, W. B. Chung, N. M. Hamburg, and J. A. Vita, "Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications," *Reviews in Endocrine and Metabolic Disorders*, vol. 11, no. 1, pp. 61–74, 2010.

[29] S. Rajagopalan and D. G. Harrison, "Reversing endothelial dysfunction with ACE inhibitors: a new TREND?" *Circulation*, vol. 94, no. 3, pp. 240–243, 1996.

[30] E. J. Henriksen, "Improvement of insulin sensitivity by antagonism of the renin-angiotensin system," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 293, no. 3, pp. R974–R980, 2007.

[31] J. Oak and H. Cai, "Attenuation of angiotensin II signaling recouples eNOS and inhibits nonendothelial NOX activity in diabetic mice," *Diabetes*, vol. 56, no. 1, pp. 118–126, 2007.

[32] G. M. Pieper, "Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin," *Journal of Cardiovascular Pharmacology*, vol. 29, no. 1, pp. 8–15, 1997.

[33] N. J. Alp, S. Mussa, J. Khoo et al., "Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression," *The Journal of Clinical Investigation*, vol. 112, no. 5, pp. 725–735, 2003.

[34] D. Tousoulis, N. Papageorgiou, E. Androulakis, K. Paroutoglou, and C. Stefanadis, "Novel therapeutic strategies targeting vascular endothelium in essential hypertension," *Expert Opinion on Investigational Drugs*, vol. 19, no. 11, pp. 1395–1412, 2010.

[35] S. R. Thomas, K. Chen, and J. F. Keaney Jr., "Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway," *The Journal of Biological Chemistry*, vol. 277, no. 8, pp. 6017–6024, 2002.

[36] G. R. Drummond, H. Cai, M. E. Davis, S. Ramasamy, and D. G. Harrison, "Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide," *Circulation Research*, vol. 86, no. 3, pp. 347–354, 2000.

[37] C. Chen, L. J. Druhan, S. Varadharaj, Y. Chen, and J. L. Zweier, "Phosphorylation of endothelial nitric-oxide synthase regulates superoxide generation from the enzyme," *The Journal of Biological Chemistry*, vol. 283, no. 40, pp. 27038–27047, 2008.

[38] F. Giacco and M. Brownlee, "Oxidative stress and diabetic complications," *Circulation Research*, vol. 107, no. 9, pp. 1058–1070, 2010.

[39] C. D. Gonzalez, M. Lee, P. Marchetti et al., "The emerging role of autophagy in the pathophysiology of diabetes mellitus," *Autophagy*, vol. 7, no. 1, pp. 2–11, 2011.

[40] N. Houstis, E. D. Rosen, and E. S. Lander, "Reactive oxygen species have a causal role in multiple forms of insulin resistance," *Nature*, vol. 440, no. 7086, pp. 944–948, 2006.

[41] J. Li, T. Ichikawa, J. S. Janicki, and T. Cui, "Targeting the Nrf2 pathway against cardiovascular disease," *Expert Opinion on Therapeutic Targets*, vol. 13, no. 7, pp. 785–794, 2009.

[42] X. Cheng, R. C. M. Siow, and G. E. Mann, "Impaired redox signaling and antioxidant gene expression in endothelial cells in diabetes: a role for mitochondria and the nuclear factor-E2-related factor 2-Kelch-like ECH-associated protein 1 defense pathway," *Antioxidants and Redox Signaling*, vol. 14, no. 3, pp. 469–487, 2011.

[43] Y. Tan, T. Ichikawa, J. Li et al., "Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo," *Diabetes*, vol. 60, no. 2, pp. 625–633, 2011.

[44] J. Zhang, J. M. Patel, Y. D. Li, and E. R. Block, "Proinflammatory cytokines downregulate gene expression and activity of constitutive nitric oxide synthase in porcine pulmonary artery endothelial cells," *Research Communications in Molecular Pathology and Pharmacology*, vol. 96, no. 1, pp. 71–87, 1997.

[45] P. Libby, P. M. Ridker, and A. Maseri, "Inflammation and atherosclerosis," *Circulation*, vol. 105, no. 9, pp. 1135–1143, 2002.

[46] J. A. Beckman, F. Paneni, F. Cosentino et al., "Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part II," *European Heart Journal*, vol. 34, no. 31, pp. 2444–2452, 2013.

[47] J. F. Keaney Jr., J. M. Massaro, M. G. Larson et al., "Heritability and correlates of intercellular adhesion molecule-1 in the Framingham Offspring study," *Journal of the American College of Cardiology*, vol. 44, no. 1, pp. 168–173, 2004.

[48] A. Festa, R. D'Agostino Jr., G. Howard, L. Mykkänen, R. P. Tracy, and S. M. Haffner, "Chronic subclinical inflammation as part of the insulin resistance syndrome: the insulin resistance atherosclerosis study (IRAS)," *Circulation*, vol. 102, no. 1, pp. 42–47, 2000.

[49] A. D. Pradhan, J. E. Manson, N. Rifai, J. E. Buring, and P. M. Ridker, "C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus," *Journal of the American Medical Association*, vol. 286, no. 3, pp. 327–334, 2001.

[50] M. B. Schulze, E. B. Rimm, T. Li, N. Rifai, M. J. Stampfer, and F. B. Hu, "C-reactive protein and incident cardiovascular events among men with diabetes," *Diabetes Care*, vol. 27, no. 4, pp. 889–894, 2004.

[51] B. B. Duncan, M. I. Schmidt, J. S. Pankow et al., "Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study," *Diabetes*, vol. 52, no. 7, pp. 1799–1805, 2003.

[52] M. A. Read, M. Z. Whitley, A. J. Williams, and T. Collins, "NF-κB and IκBα: an inducible regulatory system in endothelial activation," *Journal of Experimental Medicine*, vol. 179, no. 2, pp. 503–512, 1994.

[53] C. de Alvaro, T. Teruel, R. Hernandez, and M. Lorenzo, "Tumor necrosis factor alpha produces insulin resistance in skeletal muscle by activation of inhibitor kappaB kinase in a p38 MAPK-dependent manner," *The Journal of Biological Chemistry*, vol. 279, no. 17, pp. 17070–17078, 2004.

[54] F. Kim, M. Pham, E. Maloney et al., "Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 11, pp. 1982–1988, 2008.

[55] L. Bonfini, E. Migliaccio, G. Pelicci, L. Lanfrancone, and P. G. Pelicci, "Not all Shc's roads lead to Ras," *Trends in Biochemical Sciences*, vol. 21, no. 7, pp. 257–261, 1996.

[56] G. Pelicci, L. Dente, A. de Giuseppe et al., "A family of Shc related proteins with conserved PTB, CH1 and SH2 regions," *Oncogene*, vol. 13, no. 3, pp. 633–641, 1996.

[57] R. Sakai, J. T. Henderson, J. P. O'Bryan, A. J. Elia, T. M. Saxton, and T. Pawson, "The mammalian ShcB and ShcC phosphotyrosine docking proteins function in the maturation of sensory and sympathetic neurons," *Neuron*, vol. 28, no. 3, pp. 819–833, 2000.

[58] E. Migliaccio, S. Mele, A. E. Salcini et al., "Opposite effects of the p52(shc)/p46(shc) and p66(shc) splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway," *The EMBO Journal*, vol. 16, no. 4, pp. 706–716, 1997.

[59] G. Pelicci, L. Lanfrancone, F. Grignani et al., "A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction," *Cell*, vol. 70, no. 1, pp. 93–104, 1992.

[60] M. K. Wills and N. Jones, "Teaching an old dogma new tricks: twenty years of Shc adaptor signalling," *Biochemical Journal*, vol. 447, no. 1, pp. 1–16, 2012.

[61] E. Migliaccio, M. Giogio, S. Mele et al., "The p66(shc) adaptor protein controls oxidative stress response and life span in mammals," *Nature*, vol. 402, no. 6759, pp. 309–313, 1999.

[62] E. Y. Skolnik, A. Batzer, N. Li et al., "The function of GRB2 in linking the insulin receptor to Ras signaling pathways," *Science*, vol. 260, no. 5116, pp. 1953–1955, 1993.

[63] A. Aronheim, D. Engelberg, N. Li, N. Ai-Alawi, J. Schlessinger, and M. Karin, "Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway," *Cell*, vol. 78, no. 6, pp. 949–961, 1994.

[64] M. Ohmichi, K. Matuoka, T. Takenawa, and A. R. Saltiel, "Growth factors differentially stimulate the phosphorylation of Shc proteins and their association with Grb2 in PC-12 pheochromocytoma cells," *The Journal of Biological Chemistry*, vol. 269, no. 2, pp. 1143–1148, 1994.

[65] M. Y. M. El-Shemerly, D. Besser, M. Nagasawa, and Y. Nagamine, "12-O-tetradecanoylphorbol-13-acetate activates the Ras/extracellular signal-regulated kinase (ERK) signaling pathway upstream of SOS involving serine phosphorylation of Shc in NIH3T3 cells," *The Journal of Biological Chemistry*, vol. 272, no. 49, pp. 30599–30602, 1997.

[66] A. W. Kao, S. B. Waters, S. Okada, and J. E. Pessin, "Insulin stimulates the phosphorylation of the 66- and 52-kilodalton Shc isoforms by distinct pathways," *Endocrinology*, vol. 138, no. 6, pp. 2474–2480, 1997.

[67] C. P. Yang and S. B. Horwitz, "Taxol mediates serine phosphorylation of the 66-kDa Shc isoform," *Cancer Research*, vol. 60, no. 18, pp. 5171–5178, 2000.

[68] S. Nemoto and T. Finkel, "Redox regulation of forkhead proteins through a p66^{Shc}-dependent signaling pathway," *Science*, vol. 295, no. 5564, pp. 2450–2452, 2002.

[69] F. A. Khanday, T. Yamamori, I. Mattagajasingh et al., "Rac1 leads to phosphorylation-dependent increase in stability of the p66^{Shc} adaptor protein: role in Rac1-induced oxidative stress," *Molecular Biology of the Cell*, vol. 17, no. 1, pp. 122–129, 2006.

[70] P. Pinton, A. Rimessi, S. Marchi et al., "Protein kinase C β and prolyl isomerase 1 regulate mitochondrial effects of the life-span determinant p66^{Shc}," *Science*, vol. 315, no. 5812, pp. 659–663, 2007.

[71] M. Giorgio, E. Migliaccio, F. Orsini et al., "Electron transfer between cytochrome c and p66^{Shc} generates reactive oxygen species that trigger mitochondrial apoptosis," *Cell*, vol. 122, no. 2, pp. 221–233, 2005.

[72] P. Pinton and R. Rizzuto, "P66^{Shc}, oxidative stress and aging: importing a lifespan determinant into mitochondria," *Cell Cycle*, vol. 7, no. 3, pp. 304–308, 2008.

[73] A. Rimessi, R. Rizzuto, and P. Pinton, "Differential recruitment of PKC isoforms in HeLa cells during redox stress," *Cell Stress and Chaperones*, vol. 12, no. 4, pp. 291–298, 2007.

[74] M. Li, K. Chiou, and D. A. Kass, "Shear stress inhibition of H_2O_2 induced p66^{Shc} phosphorylation by ASK1-JNK inactivation in endothelium," *Heart and Vessels*, vol. 22, no. 6, pp. 423–427, 2007.

[75] Y. M. Yannoni, M. Gaestel, and L. Lin, "P66^{ShcA} interacts with MAPKAP kinase 2 and regulates its activity," *FEBS Letters*, vol. 564, no. 1-2, pp. 205–211, 2004.

[76] P. Francia, C. delli Gatti, M. Bachschmid et al., "Deletion of p66^{Shc} gene protects against age-related endothelial dysfunction," *Circulation*, vol. 110, no. 18, pp. 2889–2895, 2004.

[77] M. Trinei, M. Giorgio, A. Cicalese et al., "A p53-p66^{Shc} signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis," *Oncogene*, vol. 21, no. 24, pp. 3872–3878, 2002.

[78] C. Napoli, I. Martin-Padura, F. de Nigris et al., "Deletion of the p66^{Shc} longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 4, pp. 2112–2116, 2003.

[79] G. Graiani, C. Lagrasta, E. Migliaccio et al., "Genetic deletion of the p66^{Shc} adaptor protein protects from angiotensin II-induced myocardial damage," *Hypertension*, vol. 46, no. 2, pp. 433–440, 2005.

[80] O. R. Koch, S. Fusco, S. C. Ranieri et al., "Role of the life span determinant p66^{ShcA} in ethanol-induced liver damage," *Laboratory Investigation*, vol. 88, no. 7, pp. 750–760, 2008.

[81] G. Zaccagnini, F. Martelli, P. Fasanaro et al., "P66^{ShcA} modulates tissue response to hindlimb ischemia," *Circulation*, vol. 109, no. 23, pp. 2917–2923, 2004.

[82] G. Zaccagnini, F. Martelli, A. Magenta et al., "P66^{ShcA} and oxidative stress modulate myogenic differentiation and skeletal muscle regeneration after hind limb ischemia," *The Journal of Biological Chemistry*, vol. 282, no. 43, pp. 31453–31459, 2007.

[83] F. C. Franzeck, D. Hof, R. D. Spescha et al., "Expression of the aging gene p66^{Shc} is increased in peripheral blood monocytes of patients with acute coronary syndrome but not with stable coronary artery disease," *Atherosclerosis*, vol. 220, no. 1, pp. 282–286, 2012.

[84] G. G. Camici, M. Schiavoni, P. Francia et al., "Genetic deletion of p66^{Shc} adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 12, pp. 5217–5222, 2007.

[85] G. H. Kim, J. J. Ryan, and S. L. Archer, "The role of redox signaling in epigenetics and cardiovascular disease," *Antioxidants and Redox Signaling*, vol. 18, no. 15, pp. 1920–1936, 2013.

[86] A. Malhotra, H. Vashistha, V. S. Yadav et al., "Inhibition of p66^{ShcA} redox activity in cardiac muscle cells attenuates hyperglycemia-induced oxidative stress and apoptosis," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 296, no. 2, pp. H380–H388, 2009.

[87] N. Ahmed, "Advanced glycation endproducts: role in pathology of diabetic complications," *Diabetes Research and Clinical Practice*, vol. 67, no. 1, pp. 3–21, 2005.

[88] E. Schleicher and U. Friess, "Oxidative stress, AGE, and atherosclerosis," *Kidney International*, vol. 72, no. 106, pp. S17–S26, 2007.

[89] W. Cai, J. C. He, L. Zhu, X. Chen, G. E. Striker, and H. Vlassara, "AGE-receptor-1 counteracts cellular oxidant stress induced by AGEs via negative regulation of p66^{Shc}-dependent FKHRL1 phosphorylation," *American Journal of Physiology—Cell Physiology*, vol. 294, no. 1, pp. C145–C152, 2008.

[90] J. Chintapalli, S. Yang, D. Opawumi et al., "Inhibition of wild-type p66^{ShcA} in mesangial cells prevents glycooxidant-dependent FOXO3a regulation and promotes the survival phenotype," *American Journal of Physiology—Renal Physiology*, vol. 292, no. 2, pp. F523–F530, 2007.

[91] E. W.-F. Lam, R. E. Francis, and M. Petkovic, "FOXO transcription factors: key regulators of cell fate," *Biochemical Society Transactions*, vol. 34, no. 5, pp. 722–726, 2006.

[92] S. Purdom and Q. M. Chen, "Linking oxidative stress and genetics of aging with p66^{Shc} signaling and forkhead transcription factors," *Biogerontology*, vol. 4, no. 4, pp. 181–191, 2003.

[93] G. Pani, O. R. Koch, and T. Galeotti, "The p53-p66^{Shc}-Manganese Superoxide Dismutase (MnSOD) network: a mitochondrial intrigue to generate reactive oxygen species," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 5, pp. 1002–1005, 2009.

[94] T. Yamamori, A. R. White, I. Mattagajasingh et al., "P66^{Shc} regulates endothelial NO production and endothelium-dependent vasorelaxation: implications for age-associated vascular dysfunction," *Journal of Molecular and Cellular Cardiology*, vol. 39, no. 6, pp. 992–995, 2005.

[95] S. Menini, C. Iacobini, C. Ricci et al., "Ablation of the gene encoding p66^{Shc} protects mice against AGE-induced glomerulopathy by preventing oxidant-dependent tissue injury and further AGE accumulation," *Diabetologia*, vol. 50, no. 9, pp. 1997–2007, 2007.

[96] A. Natalicchio, L. Laviola, C. de Tullio et al., "Role of the p66^{Shc} isoform in insulin-like growth factor I receptor signaling through MEK/Erk and regulation of actin cytoskeleton in rat myoblasts," *The Journal of Biological Chemistry*, vol. 279, no. 42, pp. 43900–43909, 2004.

[97] A. Natalicchio, F. de Stefano, S. Perrini et al., "Involvement of the p66^{Shc} protein in glucose transport regulation in skeletal muscle myoblasts," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 296, no. 2, pp. E228–E237, 2009.

[98] E. Pagnin, G. Fadini, R. de Toni, A. Tiengo, L. Calò, and A. Avogaro, "Diabetes induces p66^{Shc} gene expression in human peripheral blood mononuclear cells: relationship to oxidative," *Journal of Clinical Endocrinology and Metabolism*, vol. 90, no. 2, pp. 1130–1136, 2005.

[99] T. Thum, D. Fraccarollo, M. Schultheiss et al., "Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes," *Diabetes*, vol. 56, no. 3, pp. 666–674, 2007.

[100] V. Di Stefano, C. Cencioni, G. Zaccagnini, A. Magenta, M. C. Capogrossi, and F. Martelli, "P66^{ShcA} modulates oxidative stress and survival of endothelial progenitor cells in response to high glucose," *Cardiovascular Research*, vol. 82, no. 3, pp. 421–429, 2009.

[101] C. Napoli, J. L. Witztum, F. Calara, F. de Nigris, and W. Palinski, "Maternal hypercholesterolemia enhances atherogenesis in normocholesterolemic rabbits, which is inhibited by antioxidant or lipid-lowering intervention during pregnancy: an experimental model of atherogenic mechanisms in human fetuses," *Circulation Research*, vol. 87, no. 10, pp. 946–952, 2000.

[102] C. Napoli, F. de Nigris, and W. Palinski, "Multiple role of reactive oxygen species in the arterial wall," *Journal of Cellular Biochemistry*, vol. 82, no. 4, pp. 674–682, 2001.

[103] C. Napoli, F. de Nigris, J. S. Welch et al., "Maternal hypercholesterolemia during pregnancy promotes early atherogenesis in LDL receptor-deficient mice and alters aortic gene expression determined by microarray," *Circulation*, vol. 105, no. 11, pp. 1360–1367, 2002.

[104] A. Boullier, D. A. Bird, M. Chang et al., "Scavenger receptors, oxidized LDL, and atherosclerosis," *Annals of the New York Academy of Sciences*, vol. 947, pp. 214–223, 2001.

[105] A. Bosutti, G. Grassi, M. Zanetti et al., "Relation between the plasma levels of LDL-cholesterol and the expression of the early marker of inflammation long pentraxin PTX3 and the stress response gene p66(ShcA) in pacemaker-implanted patients," *Clinical and Experimental Medicine*, vol. 7, no. 1, pp. 16–23, 2007.

[106] T. Minamino, H. Miyauchi, T. Yoshida, Y. Ishida, H. Yoshida, and I. Komuro, "Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction," *Circulation*, vol. 105, no. 13, pp. 1541–1544, 2002.

[107] S. V. Brodsky, O. Gealekman, J. Chen et al., "Prevention and reversal of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen," *Circulation Research*, vol. 94, no. 3, pp. 377–384, 2004.

[108] H. Matsui-Hirai, T. Hayashi, S. Yamamoto et al., "Dose-dependent modulatory effects of insulin on glucose-induced endothelial senescence in vitro and in vivo: a relationship between telomeres and nitric oxide," *Journal of Pharmacology and Experimental Therapeutics*, vol. 337, no. 3, pp. 591–599, 2011.

[109] R. J. Colman, R. M. Anderson, S. C. Johnson et al., "Caloric restriction delays disease onset and mortality in rhesus monkeys," *Science*, vol. 325, no. 5937, pp. 201–204, 2009.

[110] B. Rogina and S. L. Helfand, "Sir2 mediates longevity in the fly through a pathway related to calorie restriction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 45, pp. 15998–16003, 2004.

[111] M. Potente and S. Dimmeler, "Emerging roles of SIRT1 in vascular endothelial homeostasis," *Cell Cycle*, vol. 7, no. 14, pp. 2117–2122, 2008.

[112] S. Zhou, H. Chen, Y. Wan et al., "Repression of $p66^{Shc}$ expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction," *Circulation Research*, vol. 109, no. 6, pp. 639–648, 2011.

[113] R. R. Alcendor, S. Gao, P. Zhai et al., "Sirt1 regulates aging and resistance to oxidative stress in the heart," *Circulation Research*, vol. 100, no. 10, pp. 1512–1521, 2007.

[114] I. Mattagajasingh, C. Kim, A. Naqvi et al., "SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 37, pp. 14855–14860, 2007.

[115] A. Brunet, L. B. Sweeney, J. F. Sturgill et al., "Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase," *Science*, vol. 303, no. 5666, pp. 2011–2015, 2004.

[116] J. Luo, A. Y. Nikolaev, S. Imai et al., "Negative control of p53 by Sir2α promotes cell survival under stress," *Cell*, vol. 107, no. 2, pp. 137–148, 2001.

[117] H. Chen, Y. Wan, S. Zhou et al., "Endothelium-specific SIRT1 overexpression inhibits hyperglycemia-induced upregulation of vascular cell senescence," *Science China Life Sciences*, vol. 55, no. 6, pp. 467–473, 2012.

[118] M. A. Reddy, W. Jin, L. Villeneuve et al., "Pro-inflammatory role of MicroRNA-200 in vascular smooth muscle cells from diabetic mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 3, pp. 721–729, 2012.

[119] S. Brabletz and T. Brabletz, "The ZEB/miR-200 feedback loop-a motor of cellular plasticity in development and cancer?" *EMBO Reports*, vol. 11, no. 9, pp. 670–677, 2010.

[120] A. Magenta, C. Cencioni, P. Fasanaro et al., "MiR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition," *Cell Death and Differentiation*, vol. 18, no. 10, pp. 1628–1639, 2011.

[121] J. Rosati, F. Spallotta, S. Nanni et al., "Smad-interacting protein-1 and microRNA 200 family define a nitric oxide-dependent molecular circuitry involved in embryonic stem cell mesendoderm differentiation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 4, pp. 898–907, 2011.

[122] L. M. Villeneuve, M. Kato, M. A. Reddy, M. Wang, L. Lanting, and R. Natarajan, "Enhanced levels of microRNA-125b in vascular smooth muscle cells of diabetic db/db mice lead to increased inflammatory gene expression by targeting the histone methyltransferase Suv39h1," *Diabetes*, vol. 59, no. 11, pp. 2904–2915, 2010.

[123] S. Manca, A. Magrelli, S. Cialfi et al., "Oxidative stress activation of miR-125b is part of the molecular switch for Hailey-Hailey disease manifestation," *Experimental Dermatology*, vol. 20, no. 11, pp. 932–937, 2011.

[124] J. M. Wang, J. Tao, D. D. Chen et al., "MicroRNA miR-27b rescues bone Marrow-Derived angiogenic cell function and accelerates wound healing in type 2 diabetes mellitus," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 1, pp. 99–109, 2014.

[125] P. Fasanaro, Y. D'Alessandra, V. Di Stefano et al., "MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand ephrin-A3," *The Journal of Biological Chemistry*, vol. 283, no. 23, pp. 15878–15883, 2008.

[126] G. Zaccagnini, B. Maimone, V. Di Stefano et al., "Hypoxia-induced miR-210 modulates tissue response to acute peripheral ischemia," *Antioxid Redox Signal*, 2013.

[127] S. Greco, P. Fasanaro, S. Castelvecchio et al., "MicroRNA dysregulation in diabetic ischemic heart failure patients," *Diabetes*, vol. 61, no. 6, pp. 1633–1641, 2012.

[128] Y. Li, M. A. Reddy, F. Miao et al., "Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-κB-dependent inflammatory genes: relevance to diabetes and inflammation," *The Journal of Biological Chemistry*, vol. 283, no. 39, pp. 26771–26781, 2008.

[129] A. El-Osta, D. Brasacchio, D. Yao et al., "Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia," *Journal of Experimental Medicine*, vol. 205, no. 10, pp. 2409–2417, 2008.

[130] R. Candido, K. A. Jandeleit-Dahm, Z. Cao et al., "Prevention of accelerated atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E-deficient mice," *Circulation*, vol. 106, no. 2, pp. 246–253, 2002.

[131] R. Candido, T. J. Allen, M. Lassila et al., "Irbesartan but not amlodipine suppresses diabetes-associated atherosclerosis," *Circulation*, vol. 109, no. 12, pp. 1536–1542, 2004.

[132] D. S. Kerr, "Treatment of mitochondrial electron transport chain disorders: a review of clinical trials over the past decade," *Molecular Genetics and Metabolism*, vol. 99, no. 3, pp. 246–255, 2010.

[133] F. Paneni, P. Mocharla, A. Akhmedov et al., "Gene silencing of the mitochondrial adaptor p66(Shc) suppresses vascular hyperglycemic memory in diabetes," *Circulation Research*, vol. 111, no. 3, pp. 278–289, 2012.

[134] Y. R. Kim, C. S. Kim, A. Naqvi et al., "Epigenetic upregulation of $p66^{Shc}$ mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 303, no. 2, pp. H189–H196, 2012.

[135] T. Münzel, T. Gori, R. M. Bruno, and S. Taddei, "Is oxidative stress a therapeutic target in cardiovascular disease?" *European Heart Journal*, vol. 31, no. 22, pp. 2741–2748, 2010.

[136] N. G. Stephens, A. Parsons, P. M. Schofield et al., "Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS)," *The Lancet*, vol. 347, no. 9004, pp. 781–786, 1996.

[137] R. M. Salonen, K. Nyyssönen, J. Kaikkonen et al., "Six-year effect of combined vitamin C and E supplementation on atherosclerotic progression: the antioxidant supplementation in atherosclerosis prevention (ASAP) study," *Circulation*, vol. 107, no. 7, pp. 947–953, 2003.

[138] P. M. Kris-Etherton, A. H. Lichtenstein, B. V. Howard, D. Steinberg, and J. L. Witztum, "Antioxidant vitamin supplements and cardiovascular disease," *Circulation*, vol. 110, no. 5, pp. 637–641, 2004.

[139] H. D. Sesso, J. E. Buring, W. G. Christen et al., "Vitamins E and C in the prevention of cardiovascular disease in men: the physicians' health study II randomized controlled trial," *Journal of the American Medical Association*, vol. 300, no. 18, pp. 2123–2133, 2008.

[140] G. Bjelakovic, D. Nikolova, L. L. Gluud, R. G. Simonetti, and C. Gluud, "Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic

review and meta-analysis," *Journal of the American Medical Association*, vol. 297, no. 8, pp. 842–857, 2007.

[141] J. Stoclet, T. Chataigneau, M. Ndiaye et al., "Vascular protection by dietary polyphenols," *European Journal of Pharmacology*, vol. 500, no. 1–3, pp. 299–313, 2004.

[142] L. Hooper, P. A. Kroon, E. B. Rimm et al., "Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials," *American Journal of Clinical Nutrition*, vol. 88, no. 1, pp. 38–50, 2008.

[143] T. Szkudelski, "Resveratrol-induced inhibition of insulin secretion from rat pancreatic islets: evidence for pivotal role of metabolic disturbances," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 293, no. 4, pp. E901–E907, 2007.

[144] T. Szkudelski and K. Szkudelska, "Anti-diabetic effects of resveratrol," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 34–39, 2011.

[145] P. Palsamy and S. Subramanian, "Ameliorative potential of resveratrol on proinflammatory cytokines, hyperglycemia mediated oxidative stress, and pancreatic β-cell dysfunction in streptozotocin-nicotinamide-induced diabetic rats," *Journal of Cellular Physiology*, vol. 224, no. 2, pp. 423–432, 2010.

[146] H. Ghanim, C. L. Sia, K. Korzeniewski et al., "A resveratrol and polyphenol preparation suppresses oxidative and inflammatory stress response to a high-fat, high-carbohydrate meal," *Journal of Clinical Endocrinology and Metabolism*, vol. 96, no. 5, pp. 1409–1414, 2011.

[147] M. Lagouge, C. Argmann, Z. Gerhart-Hines et al., "Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α," *Cell*, vol. 127, no. 6, pp. 1109–1122, 2006.

[148] S. Timmers, M. K. Hesselink, and P. Schrauwen, "Therapeutic potential of resveratrol in obesity and type 2 diabetes: new avenues for health benefits?" *Annals of the New York Academy of Sciences*, vol. 1290, pp. 83–89, 2013.

[149] H. C. Williams and K. K. Griendling, "NADPH oxidase inhibitors: new antihypertensive agents?" *Journal of Cardiovascular Pharmacology*, vol. 50, no. 1, pp. 9–16, 2007.

[150] R. Cangemi, L. Loffredo, R. Carnevale et al., "Early decrease of oxidative stress by atorvastatin in hypercholesterolaemic patients: effect on circulating vitamin E," *European Heart Journal*, vol. 29, no. 1, pp. 54–62, 2008.

[151] S. John, C. Delles, J. Jacobi et al., "Rapid improvement of nitric oxide bioavailability after lipid-lowering therapy with cerivastatin within two weeks," *Journal of the American College of Cardiology*, vol. 37, no. 5, pp. 1351–1358, 2001.

[152] H. P. Friedl, D. J. Smith, G. O. Till, P. D. Thomson, D. S. Louis, and P. A. Ward, "Ischemia-reperfusion in humans. Appearance of xanthine oxidase activity," *The American Journal of Pathology*, vol. 136, no. 3, pp. 491–495, 1990.

[153] R. Butler, A. D. Morris, J. J. F. Belch, A. Hill, and A. D. Struthers, "Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension," *Hypertension*, vol. 35, no. 3, pp. 746–751, 2000.

[154] T. Gori and J. D. Parker, "Nitrate-induced toxicity and preconditioning: a rationale for reconsidering the use of these drugs," *Journal of the American College of Cardiology*, vol. 52, no. 4, pp. 251–254, 2008.

[155] S. R. Steinhubl, "Why have antioxidants failed in clinical trials?" *American Journal of Cardiology*, vol. 101, no. 10, pp. 14D–19D, 2008.

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Clinical Study

Brachial Artery Endothelial Responses during Early Recovery from an Exercise Bout in Patients with Coronary Artery Disease

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This study examined the acute endothelial responses to an exercise bout in coronary artery disease (CAD) patients. Nineteen males with CAD (63 ± 8 years) were assessed at rest and 15 minutes following a submaximal exercise bout (recovery). Brachial artery endothelial-dependent function was assessed using flow-mediated dilation (FMD). Brachial artery diameters and velocities were measured using Duplex ultrasound at baseline, and for 3 minutes following a 5-minute ischemic period. Endothelial-independent function was assessed using a 0.4 mg dose of nitroglycerin (NTG). FMD responses were unchanged from rest to recovery; however, there were 2 types of responses: negative and positive FMD responders. Post-hoc analysis revealed that positive responders had lower resting FMD compared to negative responders (3.2 ± 1.7 versus $6.0 \pm 2.5\%$, $P < 0.05$). NTG-mediated dilation was reduced in recovery (22.0 ± 5.6 versus $14.4 \pm 5.7\%$, $P < 0.001$ for rest versus recovery). In conclusion, acute endothelial-dependent responses to submaximal exercise are affected by the degree of resting endothelial dysfunction. The observation of attenuated NTG-mediated dilation during recovery is novel and warrants the investigation of possible mechanisms and clinical significance. Furthermore, it highlights the necessity of both endothelial-dependent and endothelial-independent assessments when evaluating endothelial function changes with an intervention.

1. Introduction

Patients with coronary artery disease (CAD) are characterized as having endothelial dysfunction, which describes a decreased capacity of the endothelium to elicit vasodilation [1]. The degree of endothelial dysfunction can be assessed noninvasively using a combination of techniques. Specifically, brachial artery flow-mediated dilation (FMD) provides a measure of endothelial-dependent function which has been shown to be largely mediated by the potent vasodilator nitric oxide (NO) [2] and is an accepted surrogate for coronary artery endothelial-dependent function [3]. Conversely, endothelial-independent function can be measured using an exogenous NO donor such as nitroglycerin (NTG) and is commonly assessed in combination with FMD as a control test to determine whether changes in vasodilation are attributed to the endothelial or vascular smooth muscle layers [4]. FMD has been shown to be attenuated in patients with CAD relative to healthy individuals [2, 5–7], while NTG-mediated dilation has been shown to be attenuated [5, 7, 8] or maintained [2, 6].

In patients with CAD, an attenuated FMD response is associated with an increased risk for future cardiovascular events and death [9–11]. While there is definitive evidence that chronic exercise training improves FMD in CAD patients [12–15], the FMD responses to a single bout of exercise in this population are poorly understood. Previous research in non-CAD populations has demonstrated both reductions [16–22] and no change [17, 19, 20, 22] in FMD responses during early recovery from a single bout of exercise. Postexercise reductions in FMD may indicate a period of elevated cardiovascular risk and therefore are worthy of examination in at-risk populations such as patients with CAD. Therefore, the purpose of this study was to examine the brachial

artery endothelial-dependent and endothelial-independent responses during the early recovery period (i.e., 15 minutes) following a single bout of submaximal exercise. Based on previous research in clinical populations [18, 19], we hypothesized that FMD would be transiently impaired following the acute bout of exercise, whereas NTG responses would be unchanged.

2. Materials and Methods

2.1. Participants. Nineteen males with documented CAD were recruited from the Cardiac Health and Rehabilitation Centre at the Hamilton Health Sciences General Site (Ontario, Canada). All patients were recently diagnosed as having CAD, which was defined as having at least one of the following: angiographically documented stenosis ≥50% in at least one major coronary artery; prior history of documented myocardial infarction (MI), percutaneous coronary intervention (PCI), or coronary artery bypass graft (CAGB) surgery; and positive exercise stress test determined by a positive nuclear scan, or symptoms of chest discomfort accompanied by electrocardiographic (ECG) changes of >1 mm horizontal or down sloping ST segment depression. Exclusion criteria included smoking within three months, noncardiac surgical procedure within two months, MI or CABG within two months, PCI within one month, New York Heart Association class II-IV symptoms of heart failure, documented valve stenosis, documented severe chronic obstructive pulmonary disease, symptomatic peripheral arterial disease, unstable angina, uncontrolled hypertension, uncontrolled atrial arrhythmia or ventricular dysrhythmia, insulin requiring diabetes mellitus, and any musculoskeletal abnormality that would limit exercise participation. The study protocol was reviewed and approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board, conforming to the Helsinki Declaration on the use of human subjects, and written informed consent was obtained from patients prior to participation.

2.2. Study Design. Patients attended 2 testing sessions, separated by 10 ± 6 days. The first visit involved a medically supervised exercise test to determine their peak exercise capacity. The second visit took place between the hours of 0800–1200 and involved the submaximal exercise bout and brachial artery endothelial function assessments. Prior to testing sessions, participants were instructed to fast for at least 8 hours and to abstain from exercise for 24 hours, caffeine and alcohol consumption for 12 hours, and to avoid taking NTG the morning of the visit. All testing was performed in a temperature-controlled room (23.3 ± 1.1°C).

2.3. Acute Submaximal Exercise Bout. Peak exercise capacity was assessed using a medically supervised graded exercise test to fatigue on a cycle ergometer (Ergoline, Bitz, Germany). Following a brief unloaded warmup, patients cycled at 70 rpm at a workload of 100 kpm for 1 minute, after which workload was increased by 100 kpm every minute until volitional fatigue. Expired gas was analyzed using a semiautomated metabolic cart (*V* max 229; SensorMedics Corporation, Yorba Linda, CA, USA), and oxygen uptake was determined at peak (VO_2 peak) from 20-second averages. Heart rate was monitored throughout the test using a 12-lead ECG (MAC 5500; General Electric, Freiburg, Germany).

The exercise intensities for the submaximal exercise bout were based on the peak power output achieved during the medically supervised graded exercise test. Following a 3-minute unloaded warmup on a cycle ergometer (Excalibur Sport V2.0; Lode BV, Groningen, The Netherlands), patients performed 4, 3 minutes stages at increasing intensities of 20%, 40%, 60%, and 80% of peak power output.

2.4. Brachial Artery Assessments. All rest and recovery (following the submaximal exercise bout) vascular assessments were performed in the supine position. Resting measurements were performed following 30 minutes of quiet rest, while recovery measurements were initiated 15 minutes following the cessation of submaximal exercise. Heart rate and brachial artery blood pressure were measured throughout testing using a single-lead (CC_5) ECG (model ML 123; ADInstruments Inc., Colorado Springs, CO, USA) and noninvasive hemodynamic monitor (Nexfin; BMEYE, Amsterdam, The Netherlands).

Brachial artery endothelial-dependent function was assessed using the FMD test, based on previously established guidelines [4, 23]. Duplex ultrasound (Vivid Q; GE Medical Systems, Horten, Norway) was used to capture simultaneous images of the right brachial artery (13 MHz) and blood velocity measurements (4 MHz) throughout the FMD protocol. Preocclusion images of the right brachial artery were collected 3–5 cm proximal to the antecubial fossa for 30 seconds at a frame rate of 7.7 frames·s^{-1}. A 5-minute period of ischemia was initiated by inflating a pneumatic cuff positioned on the forearm distal to the antecubial fossa to an occlusion pressure of 200 mm Hg using a rapid cuff inflator (model E20 and AG101; Hokanson, Bellevue, WA, USA). Upon cuff release, duplex postocclusion images and blood velocity measurements were collected continuously for 3 minutes. Duplex images were stored in Digital Imaging and Communications in Medicine (DICOM) format for offline analysis. End-diastolic frames, determined by the R-spike of the ECG trace, were extracted and stacked in a new DICOM file using commercially available software (Sante DICOM Editor, Version 3.0.12; Santesoft, Athens, Greece) and analyzed using semiautomated edge-tracking software (AMS (Artery Measurement System) Image and Data Analysis; Gothenburg, Sweden) as previously described [24]. Preocclusion diameters were determined from the 30-second average. Postocclusion diameters were averaged in rolling 5-cycle bins [25], and peak postocclusion diameter was defined as the maximum 5-cycle average. Absolute and relative FMD were calculated as previously described [24].

Continuous intensity weighted mean blood velocity (MBV) signals were obtained using an external spectral analysis system (model Neurovision 500 M TCD; Multigon Industries, Yonkers, NY, USA) and sampled at 100 kHz using commercially available hardware (Powerlab model ML795; ADInstruments, Colorado Springs, CO, USA). MBV was analyzed offline using LabChart 7 Pro for Windows (Powerlab

ML 795; ADInstruments, Colorado Springs, CO, USA) by correcting for the angle of insonation (all ≤ 70°). Mean blood flow was calculated by multiplying brachial artery cross-sectional area by MBV. Baseline blood flow is the average over 30 seconds, while peak blood flow is reported as the maximum 5-cycle average during the 3-minute postocclusion period. Shear rate for each bin was calculated by multiplying the MBV bin by 8 and dividing it by the corresponding reactive hyperemic 5-cycle bin. The reactive hyperemic stimulus until peak dilation was quantified as shear rate area under the curve (AUC).

Endothelial-independent function was assessed through administration of 0.4 mg sublingual spray of NTG applied 10 minutes after cuff release [4]. Longitudinal B-mode images (8 MHz) of the right brachial artery were collected at a frame rate of 22.9 frames·s^{-1} prior to administration of NTG (pre-NTG; 10 cardiac cycles) and for 10 cardiac cycles every minute post-NTG up to 10 minutes. End-diastolic frames were extracted and stacked, and peak-NTG diameters were determined from the average of 10 cardiac cycles at each minute. Absolute and relative NTG-mediated dilation was calculated as previously described [12]. We previously reported day-to-day intraclass correlations coefficients of 0.89 and 0.90 for absolute and relative FMD and NTG-mediated dilation, respectively [24].

2.5. Statistical Analysis. Statistical analyses were performed using Statistical Package for Social Science software (version 20.0; IBM Corporation, Armonk, NY, USA). Rest and recovery measures were compared using paired tests. Analysis revealed an increase in brachial artery diameters following the submaximal exercise bout, which may influence the degree of both FMD and NTG-mediated dilation. Subsequently, FMD and NTG-mediated dilation was also analyzed using an ANCOVA, as previously described [26]. Briefly, we logarithmically transformed pre- and peak diameters and calculated the change in diameter on the logged scale. This value was entered as the dependent variable in the ANCOVA with time (rest/recovery) as the fixed value and logarithmically transformed prediameter as a covariate. Covariate adjusted means for diameter change during the FMD and NTG assessments were obtained, back-transformed, and then converted to a "corrected" adjusted percentage change by subtracting 1 from the back-transformed value and multiplying it by 100. Data are presented as mean ± SD, with $P < 0.05$ considered statistically significant.

3. Results

Patient characteristics, CAD history, and medications are presented in Table 1. Peak heart rates achieved during their medically supervised graded exercise test represented 79% of their age-predicted heart rate maximum. During the submaximal exercise test, mean heart rate was 95 ± 15 bpm while peak heart rate was 122 ± 23 bpm, which is equivalent to 54 ± 22% and 78 ± 16% of their age-predicted heart rate maximums, respectively.

Endothelial function parameters are presented in Table 2. All brachial artery diameters during the FMD and NTG assessments were increased during recovery compared to rest. However, there was no difference between the FMD preocclusion and pre-NTG diameters at both rest and recovery time points, suggesting that the artery had returned to a "baseline state" prior to administration of NTG. Absolute and relative FMD and NTG responses are presented in Figure 1. FMD was unchanged from rest to recovery, while NTG-mediated dilation was attenuated during recovery compared to rest. "Corrected" FMD and NTG-mediated dilation values are presented in Table 2 and are in agreement with the results in Figure 1. Even when adjusting for preocclusion or pre-NTG diameters, "corrected" FMD was unchanged following submaximal exercise while "corrected" NTG-mediated dilation was decreased. All remaining FMD and NTG indices were unchanged with submaximal exercise. Heart rate and blood pressure indices are also presented in Table 2. Heart rate was elevated during recovery, while systolic and mean arterial pressures were lower. Diastolic blood pressure was unchanged following the submaximal exercise bout.

FMD analysis revealed two distinct groups: individuals who decreased FMD during recovery (negative responders) and individuals who increased FMD during recovery (positive responders). Post-hoc independent t-tests revealed no significant differences in patient characteristics or hemodynamics between the two groups; however, all FMD indices (absolute, relative and "corrected" FMD) at rest were lower in the positive responder group compared to the negative responder group (Table 3).

Table 1: Participant characteristics.

Variable	
Age (yr)	63 ± 8
Height (m)	1.75 ± 0.07
Weight (kg)	87.9 ± 17.0
BMI (kg·m^{-2})	28.6 ± 4.4
Cardiorespiratory assessment	
VO_2 peak (mL·kg^{-1}·min^{-1})	21.2 ± 5.2
Peak heart rate (bpm)	126 ± 21
CAD history (number)	
MI	13
PCI	13
CABG	5
Medication classification (number)	
ACE inhibitors	13
Antiplatelets	19
β-blockers	15
Calcium channel blockers	2
Statins	18
Time since CAD event (days)	156 ± 43

Data expressed as mean ± SD. ACE: angiotensin-converting enzyme. BMI: body mass index.

4. Discussion

This study provides the first examination of the endothelial responses during early recovery from an exercise bout in

Table 2: FMD, NTG, and hemodynamic indices at rest and recovery.

Variables	Rest	Recovery
FMD indices		
Preocclusion diameter (mm)	4.52 ± 0.76	4.83 ± 0.83*
Postocclusion peak diameter (mm)	4.72 ± 0.75	5.06 ± 0.85*
Time to peak RH diameter (s)	62 ± 29	64 ± 33
Preblood flow (mL·min^{-1})	49 ± 35	56 ± 34
Peak blood flow (mL·min^{-1})	351 ± 177	309 ± 139
AUC (AU)	16586 ± 13081	13855 ± 13817
"Corrected" FMD (%)	4.39 ± 2.20	4.81 ± 2.20
NTG indices		
Pre-NTG diameter (mm)	4.54 ± 0.63	4.93 ± 0.71*
Peak-NTG diameter (mm)	5.52 ± 0.69	5.63 ± 0.72†
"Corrected" NTG (%)	20.92 ± 4.46	15.14 ± 4.46*
Hemodynamic indices		
Heart rate (bpm)	57 ± 7	64 ± 8*
Systolic blood pressure (mm Hg)	126 ± 14	118 ± 11‡
Diastolic blood pressure (mm Hg)	63 ± 6	62 ± 6
Mean arterial pressure (mm Hg)	84 ± 8	81 ± 6†

Data expressed as mean ± SD. AU: arbitrary units. *$P < 0.001$ versus rest; †$P < 0.05$ versus rest. ‡$P < 0.01$ versus rest.
RH: reactive hyperemia.

Table 3: Comparison of negative and positive FMD responders.

Variable	Negative responders ($n = 9$)	Positive responders ($n = 10$)
Characteristics		
Age (yr)	64 ± 9	61 ± 7
BMI (kg·m^{-2})	28.3 ± 5.0	28.8 ± 4.0
Time since CAD event (days)	162 ± 50	150 ± 37
VO_2 peak (ml·kg^{-1}·min^{-1})	20.4 ± 4.2	21.9 ± 6.1
Submaximal peak heart rate (%HR_{max})	79 ± 18	76 ± 14
Hemodynamic indices		
Rest heart rate (bpm)	59 ± 9	54 ± 4
Rest systolic blood pressure (mm Hg)	129 ± 13	123 ± 14
Rest diastolic blood pressure (mm Hg)	65 ± 6	61 ± 6
Rest mean arterial pressure (mm Hg)	86 ± 7	82 ± 8
Endothelial indices		
Rest FMD (mm)	0.26 ± 0.11	0.14 ± 0.07*
Rest FMD (%)	6.0 ± 2.5	3.2 ± 1.7*
Rest "corrected" FMD (%)	5.9 ± 1.7	3.4 ± 1.8*
Rest NTG (mm)	1.06 ± 0.29	0.91 ± 0.17
Rest NTG (%)	23.8 ± 6.1	20.4 ± 5.0
Rest "corrected" NTG (%)	23.5 ± 4.2	20.4 ± 4.1

Data expressed as mean ± SD. %HR_{max}, percentage of age-predicted heart rate maximum. *$P < 0.05$ versus negative responders.
BMI: body mass index.

patients with CAD. While our FMD findings suggest that endothelial-dependent function is unchanged during the early stages of recovery from exercise, the observation of two distinct types of acute responses suggest that the baseline state of the endothelium may influence the acute response to a bout of exercise. Furthermore, our novel finding of attenuated NTG responses highlights an important mechanistic consideration that has been commonly overlooked by previous investigations.

To date, only 3 studies have examined the time course of brachial artery endothelial responses following exercise. Two of these investigations used an upper limb cuff during the FMD assessment and did not measure NTG-mediated dilation, which limits the ability to draw comparisons [27, 28]. The remaining study examined FMD and NTG responses at 1, 24, and 48 hours after exercise and demonstrated that the reduction in FMD observed at 1 hour after exercise returned to baseline values by 24 hours after exercise and remained stable [29]. While there is obvious merit to examining the responses during an extended period of time after exercise, there is very little information about the time course of endothelial responses during early recovery from exercise.

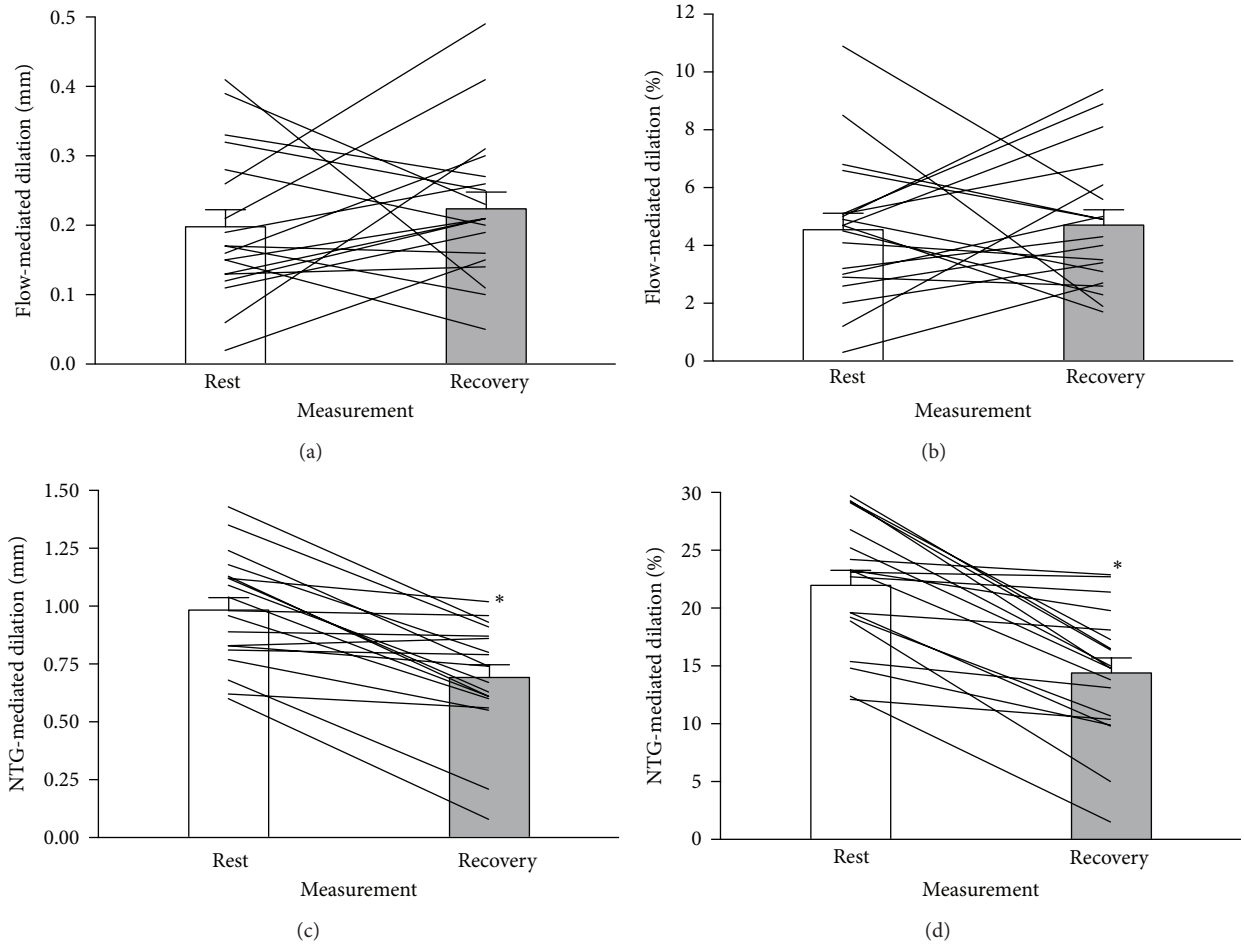

Figure 1: Absolute (mm) and relative (%) brachial artery FMD (a, b) and NTG (c, d) responses at rest (white) and 15 minutes into exercise recovery (grey). Lines represent individual patient responses. $^{*}P < 0.001$ versus rest.

The aim of the present study was to further describe the acute brachial artery endothelial responses to exercise in CAD patients by examining an earlier time point during recovery.

Contrary to our hypothesis, FMD was unchanged during early recovery from exercise, even after adjusting for the postexercise increase in brachial artery diameter. Unchanged FMD values immediately following an exercise bout have been previously reported [17, 19, 20, 22]. The absence of a change in our study is likely attributed to the large variability in individual patient responses (Figures 1(a) and 1(b)). Out of a total of 19 patients, 9 decreased FMD during early recovery from exercise, while 10 increased FMD. Conversely, all 19 patients experience reductions in NTG-mediated dilation during recovery. A comparison between negative and positive FMD responders revealed no significant difference in patient characteristics or resting hemodynamics. Specifically, positive and negative FMD responders were a similar age, exercised at a similar percentage of their age-predicted heart rate maximum during the acute exercise bout, and had similar BMI, fitness, and heart rate and blood pressure values. However, there was a difference in resting FMD such that individuals who had a lower FMD value at rest experienced a postexercise increase in FMD, while individuals who had higher FMD values at rest experienced a reduction in FMD during recovery. This is the first study to demonstrate that the direction change in FMD in response to an acute bout of exercise is dependent on the degree of baseline endothelial dysfunction. However, a previous study demonstrated that cardiac patients with endothelial dysfunction experienced positive responses to an intervention relative to the non-responses of the sample of cardiac patients with normal endothelial function [30]. Based on our novel observation of positive and negative FMD responders, we believe that additional research is required to delineate what factors influence acute endothelial-dependent responses to a bout of exercise.

To date, there are few reports of increased FMD during early recovery from an exercise bout, and the current results present data at a time point earlier than previous reports. Although it is unknown what mechanisms may mediate this response in our positive responders cohort, increased NO bioavailability [29] and elevated FMD have been observed 60 minutes following an acute bout of exercise in both healthy [31, 32] and CAD [24] individuals. NO bioavailability has been shown to be impaired in individuals with atherosclerosis [33]; therefore, an increase in NO bioavailability immediately

following exercise may explain the observed increase in FMD in our positive responders cohort with pronounced endothelial dysfunction. Conversely, several studies have documented postexercise reductions in FMD in both healthy [16–22] and clinical [18–22] populations. Exercise has been shown to increase circulation of reactive oxygen species, including superoxide anions [34, 35], which are capable of scavenging available NO, thereby decreasing its bioavailability. The postexercise reduction in FMD observed in our negative responders cohort, therefore, may be attributed to increases in oxidative stress and decreased NO bioavailability [34–36]. There is also evidence that the FMD test may not be exclusively mediated by NO; therefore, additional factors should be considered as altered sympathetic output may lead to augmented or attenuated FMD responses [37].

Evidence on endothelial-independent function in CAD patients is equivocal and suggests that NTG-mediated dilation is either impaired [5, 7, 8] or maintained [2, 6] compared to healthy controls. Our resting NTG responses of $22.0 \pm 5.6\%$ are comparable to values reported in healthy populations [5, 29, 38], which suggests that our sample had normal NTG responses during resting conditions. Few studies have examined endothelial-independent function following an acute exercise bout [19, 21, 24, 38], and presently none has demonstrated reduced NTG-mediated dilation following acute exercise. As a result, limited research is available on possible mechanisms explaining the observed responses. A 0.4 mg dose of NTG is believed to elicit the maximum obtainable diameter during resting conditions [4]; however, our data suggests that a maximum obtainable diameter may not have been reached during the resting condition since peak-NTG diameters reached during recovery were significantly larger compared to rest. If there is an upper limit for vasodilation, it was likely reached during the recovery conditions. The attenuation of relative NTG-mediated dilation during recovery could be attributed to the increased pre-NTG brachial artery diameter, and subsequent reduction in the "dilatory capacity" [39]. However, the reduction in NTG-mediated dilation was observed even after "correcting" for pre-NTG diameter, which suggests that there may be additional factors influencing NTG-mediated vasodilation. In particular, based on the observation that the NTG dose given was "submaximal" as the same dose elicited greater dilation postexercise compared to rest, the attenuation of NTG-mediated dilation during recovery may also be attributed to a shift in the dose-response relationship of vascular smooth muscle to NTG after exercise.

NTG administration permits the assessment of the vasodilatory capacity of the vascular smooth muscle; therefore, reductions in NTG-mediated dilation may be attributed to a decreased responsiveness or capacity of the vascular smooth muscle cells to elicit vasodilation. The conversion of NTG to NO occurs in the vascular smooth muscle; therefore, it is unlikely that exogenous NO was scavenged by postexercise increases in superoxide anions. However, there is evidence to suggest that the antioxidant superoxide dismutase, which neutralizes the superoxide anions, is necessary for vasodilation via the activation of soluble guanylate cyclase (sGC) and cyclic guanosine monophosphate (cGMP) [40]. A postexercise increase in superoxide anions beyond the capacity of superoxide dismutase could decrease vascular smooth muscle function by inhibiting sGC and cGMP signaling. Endothelial-independent function may also be affected by postexercise inflammation. Interleukin-1β (LI-1β) has been shown to decrease endothelial-independent dilation by decreasing sGC activation [41]. Elevated levels of IL-1β have been reported following exercise [42]. Further examinations are required to decipher the true mechanism to explain our observation of a postexercise reduction in NTG-mediated dilation.

Time to peak dilation during the FMD and NTG assessments was unaffected by submaximal exercise, suggesting that the time course of brachial artery endothelial responses remained consistent. Peak blood flow and shear rate AUC were also unchanged following acute exercise. Blood flow is regulated by downstream resistance vessels; therefore, our findings suggest that downstream vascular resistance in immediate recovery from submaximal exercise was unaffected. As shear rate AUC is used to quantify the reactive hyperemic stimulus, the absence of a change in AUC suggests a similar hyperemic stimulus across all FMD tests, which would support the validity of our observation of unchanged FMD following acute exercise. The reductions in systolic and mean arterial pressure during recovery are consistent with postexercise hypotension [43] and support the observation of postexercise increases in arterial diameters.

5. Limitations

Recovery measurements were performed approximately 45–60 minutes following the first dose of NTG. While there is evidence to demonstrate that repeated FMD tests have no effect on the subsequent test [44], no studies have determined the effect of repeated NTG doses. It is possible the first NTG dose given during resting conditions may have influenced our recovery measurements.

6. Conclusions

The combination of endothelial-dependent and endothelial-independent assessments is important because they provide a comprehensive look at the mechanisms involved in peripheral artery vasodilation. NTG assessments are used as an experimental control to measure vascular smooth muscle function. Few previous acute exercise studies have assessed both FMD and NTG responses, which in light of our current findings might limit the ability to assemble an accurate understanding of vascular regulation from previous findings. Even fewer investigations report measurements of both FMD and NTG diameters [17, 24, 32], which may play an important role in determining the dilatory capacity of the artery. We observed a mixture of positive and negative FMD responders during early recovery from submaximal exercise in patients with CAD, which is likely attributed to the degree of resting endothelial dysfunction. NTG-mediated dilation, on the other hand, was significantly attenuated in all patients during recovery. Given that this is the first study to report postexercise reductions in NTG-mediated dilation, further

research is required to delineate the mechanisms and clinical significance. Overall, this study identifies a major limitation of previous examinations of acute FMD responses to exercise interventions and highlights the necessity to perform both endothelial-dependent and endothelial-independent assessments for a more global understanding of endothelial function.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] F. Grover-Páez and A. B. Zavalza-Gómez, "Endothelial dysfunction and cardiovascular risk factors," *Diabetes Research and Clinical Practice*, vol. 84, no. 1, pp. 1–10, 2009.

[2] E. H. Lieberman, M. D. Gerhard, A. Uehata et al., "Flow-induced vasodilation of the human brachial artery is impaired in patients <40 years of age with coronary artery disease," *American Journal of Cardiology*, vol. 78, no. 11, pp. 1210–1214, 1996.

[3] B. Takase, A. Uehata, T. Akima et al., "Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease," *American Journal of Cardiology*, vol. 82, no. 12, pp. 1535–1539, 1998.

[4] M. C. Corretti, T. J. Anderson, E. J. Benjamin et al., "Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the international brachial artery reactivity task force," *Journal of the American College of Cardiology*, vol. 39, no. 2, pp. 257–265, 2002.

[5] T. Neunteufla, R. Katzenschlagerb, A. Hassana et al., "Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease," *Atherosclerosis*, vol. 129, no. 1, pp. 111–118, 1997.

[6] B. Takase, Y. Matsushima, A. Uehata, M. Ishihara, and A. Kurita, "Endothelial dysfunction, carotid artery plaque burden, and conventional exercise-induced myocardial ischemia as predictors of coronary artery disease prognosis," *Cardiovascular Ultrasound*, vol. 6, article 61, 2008.

[7] X. Y. Zhang, S. P. Zhao, X. P. Li, M. Gao, and Q. C. Zhou, "Endothelium-dependent and -independent functions are impaired in patients with coronary heart disease," *Atherosclerosis*, vol. 149, no. 1, pp. 19–24, 2000.

[8] T. Maruhashi, J. Soga, N. Fujimura et al., "Nitroglycerine-induced vasodilation for assessment of vascular function: a comparison with flow-mediated vasodilation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 6, pp. 1401–1408, 2013.

[9] E. N. Karatzis, I. Ikonomidis, G. D. Vamvakou et al., "Long-term prognostic role of flow-mediated dilatation of the brachial artery after acute coronary syndromes without ST elevation," *American Journal of Cardiology*, vol. 98, no. 11, pp. 1424–1428, 2006.

[10] M. Akcakoyun, R. Kargin, A. C. Tanalp et al., "Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events and restenosis in patients undergoing coronary stent implantation: a prospective study," *Coronary Artery Disease*, vol. 19, no. 5, pp. 337–343, 2008.

[11] Y. Kitta, J. E. Obata, T. Nakamura et al., "Persistent impairment of endothelial vasomotor function has a negative impact on outcome in patients with coronary artery disease," *Journal of the American College of Cardiology*, vol. 53, no. 4, pp. 323–330, 2009.

[12] K. D. Currie, J. B. Dubberley, R. S. McKelvie, and M. J. Macdonald, "Low-volume, high-intensity interval training in patients with CAD," *Medicine & Science in Sports & Exercise*, vol. 45, no. 8, pp. 1436–1442, 2013.

[13] P. S. Munk, E. M. Staal, N. Butt, K. Isaksen, and A. I. Larsen, "High-intensity interval training may reduce in-stent restenosis following percutaneous coronary intervention with stent implantation. A randomized controlled trial evaluating the relationship to endothelial function and inflammation," *American Heart Journal*, vol. 158, no. 5, pp. 734–741, 2009.

[14] U. Wisløff, A. Støylen, J. P. Loennechen et al., "Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients: a randomized study," *Circulation*, vol. 115, no. 24, pp. 3086–3094, 2007.

[15] R. Hambrecht, V. Adams, S. Erbs et al., "Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase," *Circulation*, vol. 107, no. 25, pp. 3152–3158, 2003.

[16] T. G. Bailey, G. K. Birk, N. T. Cable et al., "Remote ischemic preconditioning prevents reduction in brachial artery flow-mediated dilation after strenuous exercise," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 303, no. 5, pp. H533–H538, 2012.

[17] G. K. Birk, E. A. Dawson, A. M. Batterham et al., "Effects of exercise intensity on flow mediated dilation in healthy humans," *International Journal of Sports Medicine*, vol. 34, no. 5, pp. 409–414, 2013.

[18] C. L. McGowan, A. S. Levy, P. J. Millar et al., "Acute vascular responses to isometric handgrip exercise and effects of training in persons medicated for hypertension," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 291, no. 4, pp. H1797–H1802, 2006.

[19] A. Silvestro, F. Scopacasa, G. Oliva, T. de Cristofaro, L. Iuliano, and G. Brevetti, "Vitamin C prevents endothelial dysfunction induced by acute exercise in patients with intermittent claudication," *Atherosclerosis*, vol. 165, no. 2, pp. 277–283, 2002.

[20] S. A. Phillips, E. Das, J. Wang, K. Pritchard, and D. D. Gutterman, "Resistance and aerobic exercise protects against acute endothelial impairment induced by a single exposure to hypertension during exertion," *Journal of Applied Physiology*, vol. 110, no. 4, pp. 1013–1020, 2011.

[21] T. L. Llewellyn, M. E. Chaffin, K. E. Berg, and J. R. Meendering, "The relationship between shear rate and flow-mediated dilation is altered by acute exercise," *Acta Physiologica*, vol. 205, no. 3, pp. 394–402, 2012.

[22] H. Jones, D. J. Green, K. George, and G. Atkinson, "Intermittent exercise abolishes the diurnal variation in endothelial-dependent flow-mediated dilation in humans," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 298, no. 2, pp. R427–R432, 2010.

[23] D. H. J. Thijssen, M. A. Black, K. E. Pyke et al., "Assessment of flow-mediated dilation in humans: a methodological and physiological guideline," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 1, pp. H2–H12, 2011.

[24] K. D. Currie, R. S. McKelvie, and M. J. MacDonald, "Flow-mediated dilation is acutely improved following high-intensity interval exercise," *Medicine & Science in Sports & Exercise*, vol. 44, no. 11, pp. 2057–2064, 2012.

[25] R. A. Harris, S. K. Nishiyama, D. W. Wray, and R. S. Richardson, "Ultrasound assessment of flow-mediated dilation," *Hypertension*, vol. 55, no. 5, pp. 1075–1085, 2010.

[26] G. Atkinson and A. M. Batterham, "Allometric scaling of diameter change in the original flow-mediated dilation protocol," *Atherosclerosis*, vol. 226, no. 2, pp. 425–427, 2013.

[27] W. Zhu, J. Zeng, J. Yin et al., "Both flow-mediated vasodilation procedures and acute exercise improve endothelial function in obese young men," *European Journal of Applied Physiology*, vol. 108, no. 4, pp. 727–732, 2010.

[28] A. E. Tjonna, O. Rognmo, A. Bye, T. O. Stolen, and U. Wisloff, "Time course of endothelial adaptation after acute and chronic exercise in patients with metabolic syndrome," *Journal of Strength and Conditioning Research*, vol. 25, no. 9, pp. 2552–2558, 2011.

[29] O. Rognmo, T. H. Bjornstad, C. Kahrs et al., "Endothelial function in highly endurance-trained men: effects of acute exercise," *The Journal of Strength & Conditioning Research*, vol. 22, no. 2, pp. 535–542, 2008.

[30] J. G. Akar, M. O. Al-Chekakie, T. Fugate et al., "Endothelial dysfunction in heart failure identifies responders to cardiac resynchronization therapy," *Heart Rhythm*, vol. 5, no. 9, pp. 1229–1235, 2008.

[31] R. A. Harris, J. Padilla, K. P. Hanlon, L. D. Rink, and J. P. Wallace, "The flow-mediated dilation response to acute exercise in overweight active and inactive men," *Obesity*, vol. 16, no. 3, pp. 578–584, 2008.

[32] P. J. Harvey, B. L. Morris, T. Kubo et al., "Hemodynamic after-effects of acute dynamic exercise in sedentary normotensive postmenopausal women," *Journal of Hypertension*, vol. 23, no. 2, pp. 285–292, 2005.

[33] B. S. Oemar, M. R. Tschudi, N. Godoy, V. Brovkovich, T. Malinski, and T. F. Lüscher, "Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis," *Circulation*, vol. 97, no. 25, pp. 2494–2498, 1998.

[34] H. Miyazaki, S. Oh-ishi, T. Ookawara et al., "Strenuous endurance training in humans reduces oxidative stress following exhausting exercise," *European Journal of Applied Physiology*, vol. 84, no. 1-2, pp. 1–6, 2001.

[35] G. A. Tyldum, I. E. Schjerve, A. E. Tjønna et al., "Endothelial dysfunction induced by post-prandial lipemia: complete protection afforded by high-intensity aerobic interval exercise," *Journal of the American College of Cardiology*, vol. 53, no. 2, pp. 200–206, 2009.

[36] B. D. Johnson, J. Padilla, and J. P. Wallace, "The exercise dose affects oxidative stress and brachial artery flow-mediated dilation in trained men," *European Journal of Applied Physiology*, vol. 112, no. 1, pp. 33–42, 2012.

[37] M. L. Hijmering, E. S. G. Stroes, J. Olijhoek, B. A. Hutten, P. J. Blankestijn, and T. J. Rabelink, "Sympathetic activation markedly reduces endothelium-dependent, flow-mediated vasodilation," *Journal of the American College of Cardiology*, vol. 39, no. 4, pp. 683–688, 2002.

[38] T. M. Tinken, D. H. J. Thijssen, N. Hopkins, E. A. Dawson, N. T. Cable, and D. J. Green, "Shear stress mediates endothelial adaptations to exercise training in humans," *Hypertension*, vol. 55, no. 2, pp. 312–318, 2010.

[39] D. H. J. Thijssen, M. Kooijman, P. C. E. de Groot et al., "Endothelium-dependent and -independent vasodilation of the superficial femoral artery in spinal cord-injured subjects," *Journal of Applied Physiology*, vol. 104, no. 5, pp. 1387–1393, 2008.

[40] H. A. Omar, P. D. Cherry, M. P. Mortelliti, T. Burke-Wolin, and M. S. Wolin, "Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent and -independent nitrovasodilator relaxation," *Circulation Research*, vol. 69, no. 3, pp. 601–608, 1991.

[41] A. Papapetropoulos, G. Abou-Mohamed, N. Marczin, F. Murad, R. W. Caldwell, and J. D. Catravas, "Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1β," *British Journal of Pharmacology*, vol. 118, no. 6, pp. 1359–1366, 1996.

[42] A. I. Moldoveanu, R. J. Shephard, and P. N. Shek, "Exercise elevates plasma levels but not gene expression of IL-1β, IL-6, and TNF-α in blood mononuclear cells," *Journal of Applied Physiology*, vol. 89, no. 4, pp. 1499–1504, 2000.

[43] L. S. Pescatello, A. E. Fargo, C. N. Leach Jr., and H. H. Scherzer, "Short-term effect of dynamic exercise on arterial blood pressure," *Circulation*, vol. 83, no. 5, pp. 1557–1561, 1991.

[44] R. A. Harris, J. Padilla, L. D. Rink, and J. P. Wallace, "Variability of flow-mediated dilation measurements with repetitive reactive hyperemia," *Vascular Medicine*, vol. 11, no. 1, pp. 1–6, 2006.

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Review Article

Endothelium and Its Alterations in Cardiovascular Diseases: Life Style Intervention

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The endothelium, which forms the inner cellular lining of blood vessels and lymphatics, is a highly metabolically active organ that is involved in many physiopathological processes, including the control of vasomotor tone, barrier function, leukocyte adhesion, and trafficking and inflammation. In this review, we summarized and described the following: (i) endothelial cell function in physiological conditions and (ii) endothelial cell activation and dysfunction in the main cardiovascular diseases (such as atherosclerosis, and hypertension) and to diabetes, cigarette smoking, and aging physiological process. Finally, we presented the currently available evidence that supports the beneficial effects of physical activity and various dietary compounds on endothelial functions.

1. Introduction

The term endothelium was coined in the 1865 by the anatomist Wilhelm His, who differentiated the inner lining of body cavities from epithelium. Wilhelm His defined as endothelium the cell lining blood vessels, lymphatics, and mesothelial-lined cavities. This definition was later modified including only the inner cell stratum of blood vessels and lymphatics. Actually, the endothelium is considered a monocellular layer that separates all tissues from the circulating blood [1, 2]. In the 1950s and 1960s, the use of electron microscopy provided detailed and innovative ultrastructural information, revealing in endothelial cells (ECs) the presence of characteristic organelles, such as plasmalemmal vesicles, successively defined caveolae, and Weibel-Palade bodies [3, 4], and showed that the ECs are characterized by structural and functional heterogeneity. In fact, the shape and organization of cells vary across the vascular tree. Blood vessel endothelium traverses each and every tissue, but each vascular bed has unique structural and functional properties and this reflected ECs heterogeneity [5].

ECs are absent in invertebrates, cephalochordates, and tunicates but are present in the three major groups of extant vertebrates: hagfish, lampreys, and jawed vertebrates. This observation underlines that the endothelium is shared by jawless and jawed vertebrates and that it was present in the ancestor of these animals. So, it is a tissue structure conserved during the evolution of vertebrate. Yano et al. [6], for the first time, observed an unequivocal existence of organ specific properties of the endothelium confirming the structural and functional EC heterogeneity.

Developmentally, endothelium arises from mesoderm via the differentiation of hemangioblasts and/or angioblasts. However, other cell lineages may transdifferentiate into ECs and ECs into other lineages [7]. Precursor of ECs is thought to arise from the ventral floor of the dorsal aorta within the aorta-gonad-mesonephros region. Splanchnopleuric mesoderm transforms into mesenchymal cells, which differentiate into hemangioblasts. The hemangioblasts, then, become pre-ECs, which can further differentiate into either a committed haemopoietic cell line or in an EC [8].

This review will summarize and update the morphological and functional features of the endothelium and it will provide an overview of the major mechanisms participating in the alteration of endothelial functions at cardiovascular level in physiopathological states.

2. An Anatomical Overview

The anatomical structure of the endothelium is extremely simple and linear: a single layer of mesenchymal cells; despite the fact that the endothelium is an extremely complex tissue from the metabolic point of view. The EC surface in an adult human is composed approximately of 1 to 6×10^{13} cells, weighs approximately 1 kg, and covers a surface area of about 1 to 7 m^2 [9, 10]. Moreover, the ECs are generally flat, but most of the thickness of the endothelium is determined by a dynamic structure lying on its luminal surface [1]. ECs, that are typically flat, could be also plump or cuboidal in high endothelial venules (HEV) [11, 12] and EC thickness varies from less than 0.1 μm in capillaries and veins to 1 μm in the aorta [3, 13].

Endothelium may be continuous or discontinuous: continuous endothelium, in turn, may be fenestrated or nonfenestrated. The fenestrae are transcellular pores of about 70 nm in diameter extending through the full thickness of the cell and possessing a thin 5 to 6 nm nonmembranous diaphragm across their opening. The density of fenestrae varies through vascular beds [3]. In particular, continuous endothelium is characterized by ECs tightly connected to one another and surrounded by a continuous basement membrane; the subset of the continuous endothelium, defined fenestrate endothelium, has ECs permeated with holes or fenestrae, whereas the discontinuous endothelium is characterized by the presence of fenestrae, frank gaps, and a poorly formed underlying basement membrane [3]. Nonfenestrated continuous endothelium is found in arteries, veins, and capillaries of the brain, skin, heart, and lung. Fenestrated continuous endothelium, however, is characteristic of zone of high filtration or of transendothelial transport, such as capillaries of exocrine and endocrine glands, gastric and intestinal mucosa, choroids plexus, glomeruli, and renal tubules. Discontinuous endothelium is found in certain sinusoidal vascular beds, prevalently at liver level (liver sinusoidal ECs possess larger fenestrations of 100 to 200 nm in diameter, but liver showed a spatial heterogeneity) [3, 14].

Arteries and veins are both lined by continuous nonfenestrated endothelium, but endothelial junctions in arteries are tighter compared with those in veins [15]. Capillaries are characterized by flat ECs surrounded by occasional pericytes and extracellular matrix [5]. Some postcapillary venules are characterised by endothelial-lined bicuspid microscopic valves [16]. These are identical in structure, location, and orientation to the cells of the larger veins valves, with the exception that their leaflets lack fibroblasts and myofibroblasts. The ECs of postcapillary venules are rich in vesiculovacuolar organelles, particularly in the thicker portions of the blood vessels [5, 17]. Postcapillary venules are the preferred site for leukocyte and platelet trafficking, roll, and infiltration inducing endothelial activation in states of inflammation [18, 19], hypercholesterolemia, haemorrhage shock, and ischemia/reperfusion [5, 20, 21].

Table 1: High endothelial venules features with respect to normal venules, modified from Miyasaka and Tanaka (2004) [12].

	Normal venules	High endothelial venules
Endothelium	Flat	Tall and plump
Basal lamina	Thin	Thick
Perivascular sheath	Scant	Prominent
PECAM-1	Moderate	Moderate
ICAM-1	Very weak/absent	High
VE-cadherin	Moderate	Moderate

PECAM-1: platelet/endothelial cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; VE-cadherin: vascular endothelial-cadherin.

An important example of EC structural heterogeneity is found in the HEVs of secondary lymphoid organs. Anatomically, HEVs are postcapillary venules, but they are distinct from ordinary venules in several aspects [12] and also ECs in HEVs demonstrate unique structural, molecular, and functional properties compared with other ECs in the body. Each HEV is composed of a prominent perivascular sheath, a thick basal lamina, and a layer of ECs that have a plump morphology [22] and express site-specific adhesion molecules and chemokines that promote the trafficking of lymphocytes between blood and lymph node [5, 12, 23]. Some adhesion molecules are common in both normal venules and HEVs, such as platelet/endothelial cell adhesion molecule-1 (PECAM-1) (also known as CD31), whereas certain molecules are expressed uniquely in HEVs. In particular, peripheral lymph nodes HEVs abundantly express highly glycosylated and sulphated forms of sialomucins, including glycosylation-dependent cell-adhesion molecule 1, CD34, podocalyxin, endoglycan, and endomucin. With respect to normal venules, numerous sialomucins are expressed also in HEVs, but in a glycosylated form. HEVs express also high levels of lymphoid chemokines, although normal venules generally do not, with some exceptions, such as intestine and skin venules [12]. At the ultrastructural level, HEVs are characterized by a prominent Golgi complex, abundant polyribosomes, and rough endoplasmic reticulum, indicating an intense biosynthetic activity not observed in flat ECs. They also contain many membrane-bound vesicular structures, multivesicular bodies, Weibel-Palade bodies, and a variety of dense bodies, indicating that HEVs are involved in secretion. Actually, very few HEV-specific markers are known: the best marker currently available is MECA-79 [11], which stains all HEVs within lymphoid tissues and does not react with postcapillary venules or large vessels in spleen, thymus, or nonlymphoid tissues [24]. HEVs showed also "spot-welded" junctions between high ECs [11, 25] that differ from the tight junctions characteristic of capillary and arterial endothelium, but are similar to the "nonoccluding" junctions found in normal postcapillary venules. These junctions probably facilitate the passage of lymphocytes between adjacent high ECs allowing massive lymphocyte emigration in HEVs [11] (Table 1).

It is important to remember that also heart contains several endothelial compartments, including ECs of coronary arteries, capillaries, and endocardium. These subtypes of ECs differ in developmental origin, structure, and function. Endocardial ECs are larger than other types of ECs and possess many microvilli, which project into the heart cavity [5]. The relative abundance of gap junctions, such as connexins, expressed by endocardium but not by EC of myocardial capillary is due to the fact that the endocardium has more intercellular clefts, gap junctions, and few vesicles [5, 26]. The ECs of coronary arteries are similar in structure and function to ECs of other arteries in the body; whereas capillaries in the heart possess a continuous endothelium and the distance between the capillary EC and the nearest cardiomyocyte is about 1 μm leading to an optimal diffusion of oxygen and nutrients between blood and underlying cells. Like the endocardium, ECs of myocardial capillary are involved in reciprocal signalling with cardiomyocytes [5]. Moreover, cardiac microvascular ECs have been shown to promote cardiomyocyte survival [27]. In the endocardium the expression of von Willebrand factor and endothelial nitric oxide synthase (eNOS) is higher with respect to myocardial microvessels [28, 29] and eNOS is greatly concentrated in the Golgi body, whereas in myocardial capillary ECs, eNOS is more diffusely distributed in the cytoplasm [5, 30].

At cardiac valve level, there are two most prominent cellular components: the valve endothelial cells (VECs) and the interstitial cells. The VECs reside along the entire surface of the cusps or leaflets and are in continuity with endocardium, aorta, and pulmonary artery. The valve interstitial cells, however, make up the collection of mesenchymal cells that reside within specialized extracellular matrix subcompartments, fibrosa, spongiosa, and ventricularis/atrials layers. An extensive study of human VECs, on specimens ranging from 3 weeks gestation to 10 years of age, showed their progressive elongation and flattening on the ventricular side of the cusps, whereas VECs along the arterial side appeared cuboidal. ECs lining cardiac valves exhibit many of the same properties of ECs [31].

3. Morphological and Structural Features of Endothelium

The membrane of ECs is made of a double layer of phospholipids separated by water compartments and crossed by complex proteins that work as receptors or channels. ECs possess various contractile proteins, such as actin, myosin, and tropomyosin, that allow motor activities [32]. Some of these proteins are organized in a cortical web that surrounds the internal surface of the sarcolemma and defined ECs shape and elasticity. The junctions associated to actin filament, defined FAU system, are at the intercellular level and their contraction and relaxation control the dimension and hence the shape of the intercellular space, modulating the passage of solutes and macromolecules between blood and subendothelial space. The FAU system is closely related to the intercellular adhesion molecules, especially with vascular endothelial (VE) cadherin, maintaining a balance between adhesive and contractile forces [32].

ECs have three surfaces: cohesive, adhesive, and luminal. The cohesive surface adjoins ECs with each other and facilitates transport processes and consists of specialized intercellular junctions: gap, tight, or adherent junctions and syndesmos [33, 34]. The adhesive surface of ECs adheres to basal lamina and, finally, the luminal side of the vascular endothelium consists of molecules and specific binding proteins regulating trafficking of circulating blood cells [34].

ECs and their nuclei are aligned in the direction of blood flow in straight segments of arteries, but not at branch points [3, 35, 36]. When blood flow increases and so also shear stress, the ECs are flattened and aligned in the direction of the flow; whereas when blood flow decreases the ECs increase their volume losing the alignment and looking like cobble stones paving [32]. The ECs are, in fact, sensitive to changes of the intravascular tension and may increase their stiffness after the increment of the intravascular pressure.

Bevilacqua et al. [37] identified the first inducible endothelial cell-specific leukocyte adhesion molecule (ELAM-1), later designated as endothelial cell-selectin (E-selectin) [7, 38]. Considering the potential ECs markers, PECAM-1 is, in addition, expressed in monocytes; thrombomodulin as well as in ECs, is also expressed in keratinocytes, trophoblasts and leukocytes and VE-cadherin is expressed also in trophoblasts and fetal stem cells. Moreover, it is known that ECs may express also vascular endothelial growth factor (VEGF) that plays a key role in the generation and maintenance of endothelial fenestrae [3] and eNOS, whose expression increases after higher shear stress, inducing the parallel sustained increase in the production of nitric oxide (NO) [1, 39]. This free radical is the key endothelium-derived relaxing factor that plays a pivotal role in the maintenance of vascular tone and reactivity. In addition to being the main determinant of basal vascular smooth muscle tone, NO acts to negate the actions of potent endothelium-derived contracting factors, such as angiotensin II and endothelin-1 (ET-1). In addition, NO serves to inhibit platelet and white cell activation and to maintain the vascular smooth muscle cells in a nonproliferative state (Figure 1) [40].

Endothelium is also the source of the potent vasoconstrictor peptide ET-1. First isolated, purified, and sequenced in 1988 [41], in health, the production of ET-1 is minimal and it is effectively opposed by NO and other endothelium derived vasodilators [42]. The circulating level of this short peptide was quickly determinant in humans and it was reported that, in most cardiovascular diseases, circulating level of ET-1 was increased [43]. Our research group observed a significative increase of ET-1 expression at aortic level in different animal models: rats with a severe nephrotoxicity induced by cyclosporine A [44, 45], rats with vascular damage-induced by nicotine [46], and in atherosclerotic mice (ApoE-deficient mice) [47]. Molecular data and *in vitro* and *in vivo* findings [48–50] have indicated that angiotensin II can turn on transcription of the precursor of ET-1 gene and biosynthesis of ET-1 in different cell types, including cultured vascular smooth muscle cells [51, 52] and ECs by acting on

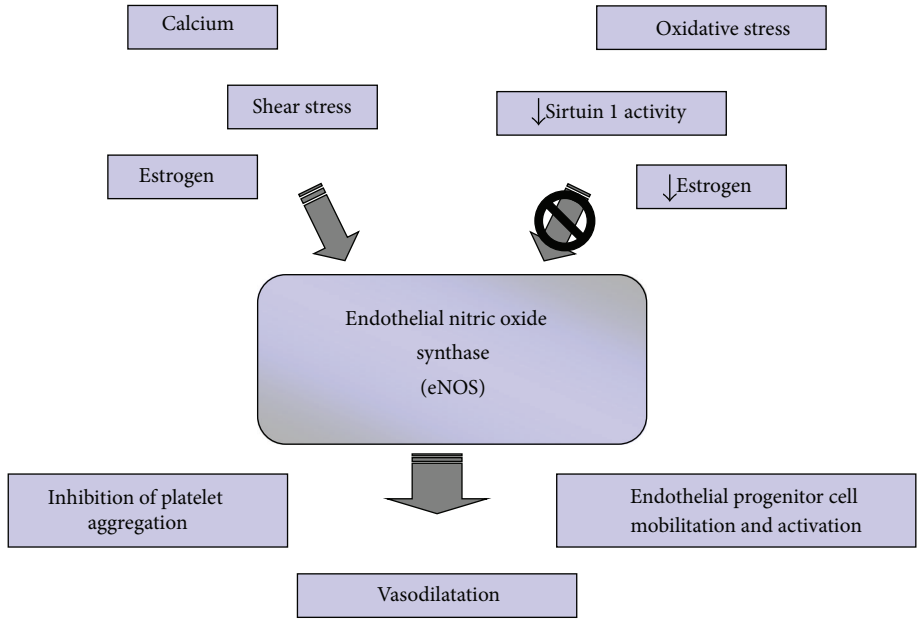

Figure 1: The activation of endothelial nitric oxide synthase (eNOS) may be induced by shear stress, estrogens, or increase of intracellular Ca^{2+} concentration and may be inhibited, mainly during aging physiopathological process, by oxidative stress and reduction of Sirtuin 1 activity and of estrogens level. In healthy endothelium, eNOS plays multiple functions, such as nitric-oxide vasodilation and inhibition of platelet aggregation and may induce endothelial progenitor cells activation and functions.

angiotensin II type 1 receptors. ET-1 might be also involved in the cardiovascular damage because it was found to contribute to hypertrophic response to angiotensin II [50, 53, 54].

Platelet-selectin (P-selectin) is expressed not only in ECs but also in megakaryocytes; it is stored intracellularly in Weibel-Palade bodies and it is expressed preferentially in postcapillary venules [3, 55]. Unlike E-selectin and P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are expressed in many vascular and nonvascular cell types. Constitutive expression of cell surface VCAM-1 in mice is generally lower than that of ICAM-1, with the exception of heart, where both are equally expressed, and brain in which VCAM-1 density is 4-fold higher with respect to ICAM-1 [3]. It is well known that the expression of ICAM-1 and VCAM-1 is strictly connected and correlated to atherogenesis [2, 3, 56].

The superoxide dismutase (SOD) enzymes are also a key determinant of NO bioavailability since SOD competes with NO for superoxide in a diffusion-limited manner, thus attenuating the formation of peroxynitrite and, indirectly, improving NO bioavailability [57, 58]. Indeed, in mice with a genetic ablation of the cytosolic isoform SOD1, endothelial dysfunction was associated with increased superoxide and peroxynitrite levels compared with wild type controls. Furthermore, exogenously added SOD was able to partially restore endothelium-dependent vasodilation in an eNOS-dependent manner [58, 59]. In addition, overexpressing the mitochondrial isoform SOD2, specifically in the endothelium of streptozotocin-induced diabetic mice, prevented diabetic retinopathy and superoxide-mediated oxidative stress [60]. These data clearly demonstrate the important role of SOD that plays a role in balancing oxidative stress through removal of superoxide and thereby maintaining NO levels [58].

Endothelial expression of tissue factor has been reported in certain pathologic conditions, including tumors [61], atherosclerosis [62], sickle cell anemia [63], sepsis, and cardiac allograft rejection [64]. It is possible that under these conditions endothelial tissue factor plays an important pathogenic role and that an understanding of its transcriptional regulation would provide new insights into mechanisms of disease [2].

3.1. Cellular Junctions. Two main types of intercellular junctions are recognized in ECs: tight junctions, defined also as zona occludens, and adherens junctions, also termed as zona adherens [3, 65, 66]. The junctional composition of intercellular clefts varies across the vascular tree. The tight junctions, which are usually found at the apical region of the intercellular clef, impart two functions in the cell: a barrier function, regulating the permeability of solutes between adjacent cells, and a function controlling the lateral diffusion of proteins within the lipid bilayer [67–69]. The ECs of large artery display a well-developed system of tight junctions due to their conduit function and their exposure to high rates of pulsatile blood flow, whereas in the arterioles the junctions are tighter with respect to capillaries and are quite loose in venules [3]. The extracellular membrane-bound components of the tight junctions are formed by proteins from three families: claudins, occludins, and junction adhesion molecules [70–73].

The identification of several components of adherens junctions in ECs helps in the understanding of the complex

role of these structures not only in maintaining cell-to-cell adhesion but also in transferring intracellular signals. VE-cadherin is an endothelial-specific adhesion protein at adherens junctions that interacts with several signalling partners inducing contact inhibition of growth and decrease in permeability.

Several pathological conditions have been associated with altered junction organization including chronic inflammation, atherosclerosis, or tumor angiogenesis. In these cases, altered junctions are likely the consequence of vascular injury or EC activation and retraction [74].

3.2. Caveolae. In physiological condition, there are various ways of transporting plasmatic molecules through the endothelial barrier: (i) intercellular unions that generally act as filters controlled by the hydrostatic pressure that allow the passage of water and dissolved substances; (ii) vesicles formed from the caveolae that help the passage of macromolecules through the cell membrane and cytoplasm; (iii) transcellular channels usually formed from various caveolae that connect opposite sides of the cell membrane. In particular, through caveolae, the endothelium regulates the passage of fluid and macromolecules between the vascular and cellular compartments; when this way fails, there is the formation of edema [32].

Caveolae are 70 nm membrane-bound, flask-shaped vesicles that are usually open to the luminal or abluminal side and are occasionally free in the cytoplasm [3]. Caveolae occupy between 5% and 10% of the total EC surface [32, 75]. The number of caveolae is the highest in continuous nonfenestrated endothelium, particularly at heart, lung, and skeletal muscle levels [3], whereas in the blood brain barrier the caveolae are rarely found [15]. It is important to remember that caveolae are present also in non-ECs.

There are three caveolin (cav) isoforms, cav-1, cav-2, and cav-3. Cav-1 and cav-2 are ubiquitously expressed, whereas cav-3 is specific for striated muscle. Cav-1 and cav-3 have a conserved cav scaffolding domain, which is bound by many membrane proteins, such as G proteins, tyrosine kinase receptors, and eNOS [76, 77]. Cav-1 and cav-2 are expressed in most cell types including all cell types of the cardiovascular system, while cav-3 is expressed prevalently in cardiac and skeletal muscle [78].

Cav-1 is the main caveolae found at the endothelial level [58]; various EC signalling molecules localize in caveolae and are modulated by direct interaction with cav-1 [58, 79]. NO production, by eNOS, is tightly regulated by enzyme interaction with cav-1 [80, 81]. Cav-1 has been shown to directly bind eNOS and inhibit eNOS-derived NO release under physiological conditions [82]. On the contrary, for optimal NO release, eNOS must reside in caveolae microdomains [58]. Interestingly, also members of the transient receptor potential channel (TRPC) family reside in caveolae [81, 83, 84].

Our previous study demonstrated that the genetic deletion of cav-1 in mice results in total absence of endothelium-derived hyperpolarizing factors- (EDHF-) mediated vasorelaxation altering calcium (Ca^{2+}) entry and disregulating the expression and caveolar location of connexins and myoendothelial and vascular homocellular gap junction components [81]. EDHFs, including NO, carbon monoxide, hydrogen sulphide, lipoxygenase, and others, regulate cellular hyperpolarization decreasing Ca^{2+} influx, either by reducing the open probability of cave channels or the cav channel-dependent activation of the sarcoplasmic reticulum, which induces relaxation of vascular smooth muscle cells [85, 86].

Cav-2 is expressed in ECs, smooth muscle cells, skeletal myoblasts, fibroblasts, white adipocytes, lung tissue, and pancreatic islets. When compared to human cav-1, cav-2 was determined to be roughly 38% identical and 58% similar to a conserved region of eight identical aminoacids. It is important to underline that cav-2 is able to directly bind cholesterol without cav-1 interaction [87].

However, cav-3 is roughly 64% identical to cav-1 and can form homooligomeric complexes with itself and does not require cav-1 to drive caveolae formation [88]. In cardiomyocytes, eNOS localizes to caveolae binding to cav-3. The colocalization of cav-3 and eNOS may facilitate both eNOS activation by cell surface receptors and NO release at the cell surface for intercellular signaling [89, 90].

3.3. Transient Receptor Potential Channel. There are six TRPC proteins in humans, but more TRPCs may arise through heteromerization among TRPCs and other types of transient receptor protein. TRPCs support endothelial functions like vascular regeneration, increased permeability, and endothelium-derived NO-mediated vasorelaxation playing a central role in the control of vascular smooth muscle cell tone, endothelial permeability, and platelet function. Some of these ion channels are relatively Ca^{2+} selective but many are nonselective cationic channels with permeability to Ca^{2+}, sodium (Na^{+}), and potassium (K^{+}). In a few instances they are Ca^{2+}-impermeable or permeable also to magnesium and other cations (Figure 2). All of the TRPCs are reported to be expressed in blood vessels, especially in ECs and vascular smooth muscle cells. They are functionally important, but almost not critical for the development or maintenance of a physiological vasculature [91].

A careful comparison of EC responses from different TRPC-deficient mice with respect to wild type mice permit to identify which channel(s) is/are essential for modulating the changes in vascular permeability [92].

Freichel and colleagues [93] observed, in primary aortic ECs of $TRPC4^{-/-}$ mice, that TRPC4 is part of the Ca^{2+} influx signal transduction pathway regulating vascular tone. However, TRPC5 might also be involved in this process; in fact, the treatment of bovine aortic ECs with siRNA against TRPC5 prevented NO-induced Ca^{2+} entry and so decreased endothelium-dependent NO vasorelaxation [94]. A TRPC5 and 6 activation cascade have been shown to take part in the regulation of ECs migration [92, 95]. However, inhibition of TRPC3 activity by protein kinase G-dependent phosphorylation was observed to protect ECs from the detrimental effect of excessive NO and Ca^{2+} [92, 96, 97]. In particular, TRPC3 channels have been suggested to serve as redox sensors which monitor oxidative stress in ECs. However, TRPC3 may not be

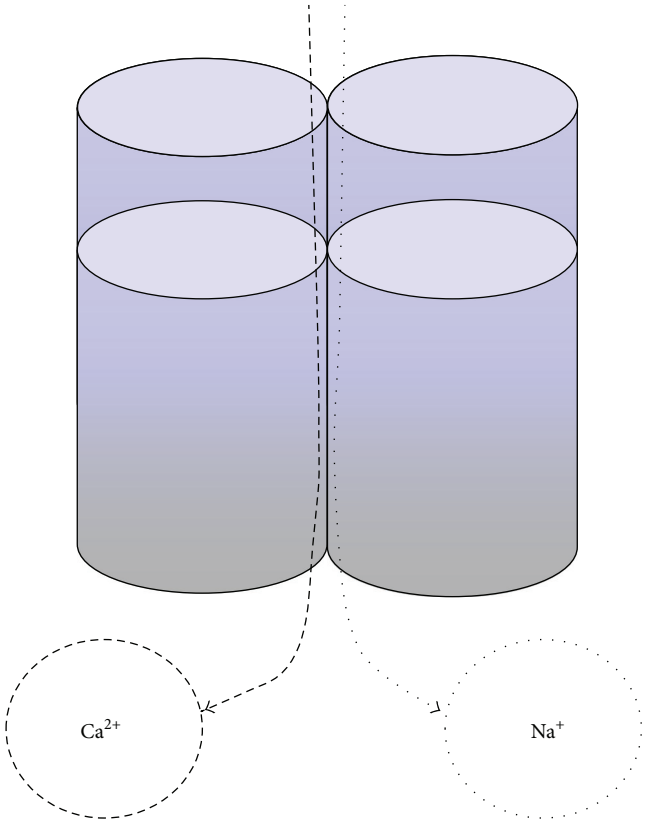

Figure 2: Transient receptor potential channels (TRPCs) clustered around a central ion pore enabling influx of calcium and sodium ions. Ca^{2+}: calcium; Na^{+}: sodium.

acting alone in this process; in fact the heteromeric channels composed of TRPC3 and 4 form redox-sensitive channels both in native ECs and when being heterologously expressed in human embryonic kidney 293 (HEK293) cells [98, 99].

Nevertheless, the exact role of these channels is still under study. TRPCs may therefore be attractive drug targets to tackle physiopathological states; therapeutic approaches to modulate activation of specific TRPCs are likely to have an important impact in reducing tissue damage in a number of diseases resulting from oxidant stress including ischemia/reperfusion injury, hypertension, edema, bleeding disorders, and diabetes [92, 99].

4. Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are bone marrow-derived stem cells that differentiate into functional ECs [100–103]. EPCs are mobilised from the bone marrow into the peripheral blood in response to tissue ischemia or injury [104]. EPCs migrate to vascular or tissue injury sites, contributing significantly to reendothelialization and neovascularization and hence tissue repair [105–108] and differentiate into ECs [109]. However, a reduction in the number of circulating EPCs may also result in the impaired reendothelialization of eroded atherosclerotic plaque and thus lead to a greater propensity for thrombosis and vascular occlusion. Several lines of evidence indicate that EPCs constitute an important endogenous system that maintains endothelial and vascular integrity [103].

Moreover, EPCs may play an important role in maintaining an intact and functional endothelium in mature blood vessels and the alteration of this function leads to abnormal vasoreactivity [101]. It was observed by Vasa and colleagues [110] that the number and migratory activity of circulating EPCs decreased in patients with risk factors for coronary artery disease, suggesting that a decrease in the EPCs level may contribute to impaired vascularization. EPCs have been also implicated in postischemic neoangiogenesis [111, 112] and are ideal candidates for vascular regeneration [113, 114]. De Ciuceis and colleagues [115] observed that EPCs and capillary density were reduced in obese subjects, but pronounced weight loss induced by bariatric surgery significantly increased EPCs and seems to induce an almost complete regression of microvascular fibrosis, while not improving capillary rarefaction. Further clinical studies are needed to amplify the knowledge about EPC mobilization and function and for the establishment of EPC measurements as prognostic markers in cardiovascular diseases.

5. Endothelial Cells Functions

The endothelium exerts its function in maintaining vascular homeostasis through the balanced release of a number of autocrine and paracrine substances in response to physical, biological, and chemical stimuli [1]. In fact, it is known that the endothelium plays an important role in many physiological functions, including the control of vascular tone, blood cell trafficking, innate and adaptive immunity, and hemostasis [3]. Endothelium is capable of producing vasoactive factors, such as vasodilators and vasoconstrictors, procoagulants and anticoagulants, inflammatory and anti-inflammatory factors, fibrinolytics and antifibrinolytics, oxidizing and antioxidizing, and other factors [32, 116, 117].

The endothelium regulates vascular tone via responding to a variety of stimuli [118]. This process involves a complex interplay between intracellular receptors, the synthesis, and then the release of a variety of endothelium-derived relaxing and constricting substances [119, 120].

Passage of leukocytes from blood to underlying tissue through the endothelial layer involves a multistep adhesion pathway that includes rolling, initial attachment, arrest, and transmigration. These steps take place prevalently in postcapillary venules. In particular, rolling is mediated primarily by interactions between leukocyte carbohydrate-based ligands and endothelial E- and P-selectin and firm adhesion by interactions between leukocyte integrins and ICAM-1 and VCAM-1. There are two pathways for the passage of leukocytes through the endothelial layer: they may pass between ECs, paracellular route, or they may pass through the EC itself, defined as transcellular route [17, 121, 122]. The molecular basis of transmigration is actually controversial, but it is known that it involves CD99, PECAM-1, and junctional adhesion molecule-1 [3, 123, 124].

Another common function of the endothelium is the capacity to maintain blood in a fluid state limiting clot formation when there is a breech in the integrity of the vascular wall and the endothelium serves as a borderline between the coagulation factors circulating in the blood and the primary initiator of coagulation within the vascular wall [86, 125, 126].

ECs express tissue factor pathway inhibitor, heparan, thrombomodulin, endothelial protein C receptor (EPCR), tissue-type plasminogen activator, ecto-ADPase, prostacyclin, and NO, as anti-coagulant factors, whereas ECs may synthesize tissue factor, plasminogen activator inhibitor-1, von Willebrand factor, and protease activated receptors (PARs), as procoagulant factors [3, 127]. EPCR is expressed predominantly in large arteries and veins [128], whereas thrombomodulin is highly expressed in blood vessel types of every calibre in all organs, with the exception of the brain, where its expression is low [3, 129]. The differential distribution of procoagulants and anticoagulants in the vessel tree suggests and confirms the endothelial heterogeneity and that ECs from different sites of the body use site-specific procoagulants or anticoagulant factors to balance specific and local haemostasis [7].

Other important functions of the endothelium are to regulate the transport of liquids across the semipermeable vascular endothelial barrier [92, 125] and function as a protective biocompatible barrier between all tissues and the circulating blood, hence modulating the bidirectional passage of macromolecules and blood gases to and from tissues and blood [41]. Importantly, these properties vary both in space and time, once and again giving rise to the phenomenon of EC heterogeneity [2, 130]. In Figure 3 the main functions of endothelium are summarized.

Given the critical role of these mechanisms in which ECs are the key factors, the deregulation of the endothelial balance, defined endothelial dysfunction, leads to the pathogenesis of many diseases including atherosclerosis, hypertension, sepsis, and some inflammatory syndromes [1, 40].

6. Endothelial Dysfunction

As described in the previous paragraphs, the endothelium is an emergent and complex system. It is multifunctional, highly distributed in space, and has an enormous behavioural repertoire; in fact EC dysfunction is not restricted anatomically to a single organ or limited in a singular disease mechanism [3, 7]. The ECs represent a powerful organizing system in human health and disease, also because they are involved in numerous pathological states either as primary determinants of physiopathology or as victims of collateral damages [5, 7].

Endothelial dysfunction disrupts the mechanism of vascular homeostasis regulation predisposing the vessel wall to vasoconstriction, leukocyte adhesion, platelet activation, oxidative stress, thrombosis, coagulation, and inflammation hence leading to the pathogenesis of cardiovascular diseases [40, 131].

Early description of EC dysfunction focused on structural changes or loss of anatomical integrity, particularly in the context of atherosclerosis: Ross and Glomset [132] proposed, in 1973, a response-to-injury hypothesis to explain the lesions of atherosclerosis. Then, Bevilacqua et al. [37] employed the term EC dysfunction to describe hyperadhesiveness of the endothelium to platelets.

It is important to underline that EC may be activated without being dysfunctional [7] and that endothelium is highly active and constantly sensing and responding to alterations of the local extracellular environment [133]. The term "activation" reflects the capacity of ECs to perform new functions without evidence of cell injury or dysfunction [134].

It is well known that the intact endothelium may actively contribute to disease initiation and/or progression. The transition between EC function and dysfunction is not always clear. EC dysfunction usually arises from otherwise adaptive responses that are now excessive, sustained, or spatially and/or temporally misplaced [7]. Moreover, the endothelium is heterogeneous in its response to physiopathological stimuli [133].

The concept of EC activation first arose from *in vitro* studies demonstrating the ability of well-defined stimuli to induce the expression of the so-called "activation antigens" on the surface of ECs; actually, P-selectin is considered a marker of EC activation [135].

In conclusion, the endothelium is highly plastic and thus amenable to therapeutic modulation, in establishing a dialogue with the underlying tissue and so it provides a possible direct line of communication with every organ in the body. The goal in treating the endothelium is not to reset the switch, but rather to fine-tune and recalibrate the EC, nudging back to their physiological state [133]. Given that EC phenotypes vary according to time and location in the vascular tree, in both health and disease states, it is essential to modulate therapy to specific vascular beds.

In the following paragraphs, the main EC dysfunction observed at cardiovascular level in the pathological states of atherosclerosis, hypertension, diabetes, and cardiac valvular degeneration or induced by age or cigarette smoking was summarized. Successively, we will briefly present a possible emerging prevention/treatment of ECs dysfunction through a healthy lifestyle.

6.1. Atherosclerosis. Endothelial dysfunction is known to be implicated in the pathogenesis and clinical course of all known cardiovascular diseases [136, 137], occurs in response to cardiovascular risk factors, and precedes the development of atherosclerosis [42, 138, 139]. Endothelial dysfunction actively participates in the process of lesion formation promoting the early and late mechanisms of atherosclerosis [32, 40, 42], determining increase in EC permeability, upregulation of adhesion molecules, chemokine and cytokine secretion, and leukocyte adherence, enhanced oxidized-low density lipoprotein (ox-LDL), platelet activation, and vascular smooth muscle cell proliferation and migration. In addition, endothelial dysfunction is not only the initial stage of the development of atherosclerotic disease that generates

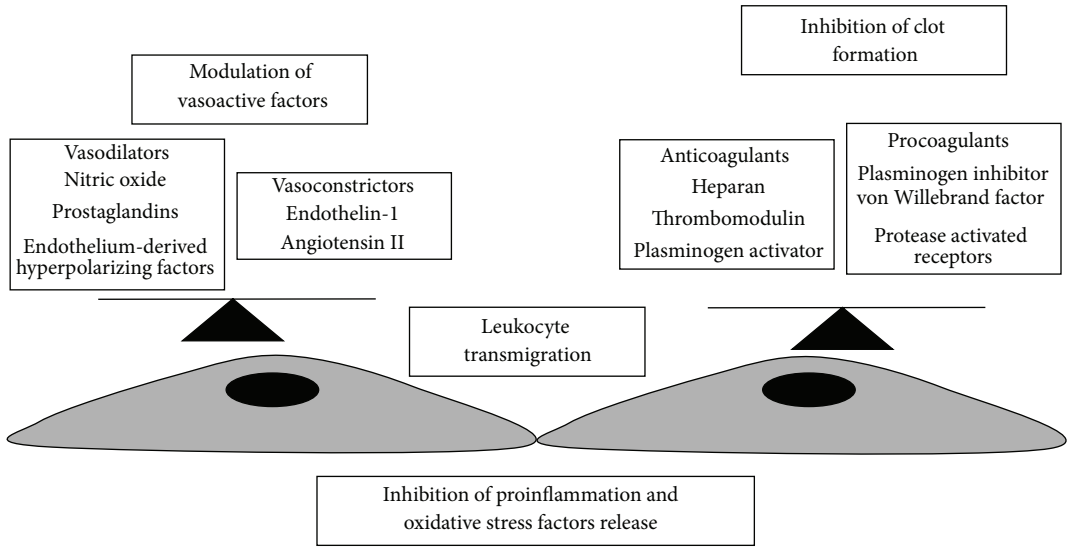

Figure 3: Endothelial functions in physiological conditions. The net balance of endothelium-derived vasodilators and vasoconstrictors or anticoagulants and procoagulants along with inhibition of proinflammatory and oxidative stress factors release and leukocyte adhesion and transmigration maintain a healthy vascular homeostasis.

plaque formation, but it also can cause plaque growth leading to vascular complications. For all these reasons, endothelial dysfunction is one of the principal mechanisms in atherosclerotic diseases [32].

The endothelial injury, activation, and dysfunction caused by ox-LDLs in the pathogenesis of atherosclerosis are exerted via the activation of lectin-like ox-LDL receptor-1 (LOX-1) activation [100, 140]. LOX-1, initially identified as the major receptor for ox-LDL in ECs, can also be expressed in macrophages and smooth vascular muscle cells [101, 141–143]. LOX-1 has the ability to bind damaged or apoptotic cells, activated platelets, advanced glycation end products, and pathogenic organisms [144, 145] and so it may play a role in initiating and potentiating the early steps of atherogenesis. However, elevated LOX-1 expression is observed not only in both initial and advanced atherosclerotic lesions [101, 143, 146, 147] but also during other cardiovascular injuries, such as hypercholesterolemia, hypertension, obesity, and diabetes [140, 148]. Ox-LDL, via upregulation of LOX-1 mediated by angiotensin II and ET-1, induces monocyte adhesion to the endothelium via enhanced expression of P-selectin, ICAM-1, and VCAM-1 [149, 150]. Receptor activation also results in monocyte chemoattractant protein-1 expression, promoting monocyte migration into the intima [101, 151]. However, ox-LDL uptake by LOX-1 also mediates EC apoptosis, potentially via nuclear factor (NF)-κB activation [101, 152] and resulting in direct vascular denudation and injury that may trigger or enhance the inflammatory and oxidative stress reactions. Ox-LDL binding to LOX-1 induces an increase production of intracellular reactive oxygen species (ROS), apoptosis of vascular smooth muscle cells, and modulation of matrix metalloproteinase activity, all factors/conditions that compromise and alter the atherosclerotic fibrous cap. Moreover, activated platelets may interact with the surrounding endothelium via LOX-1 [153] promoting the release of ET-1 from ECs, stimulating the generation of ROS that inactivates NO [145] and so starting a vicious circle that potentially ends in vascular occlusion and ischemic insult.

ECs are involved in the atherogenic process also through PAR expression. EC PAR-1 activation promotes adhesivity toward monocytes via the induction of NF-κB, which in turn promotes ICAM-1 expression [101, 154]. PAR-1 and PAR-2 activity induces discharge of Weibel-Palade bodies and endothelial storage granules containing the adhesion molecule P-selectin and von Willebrand factor, promoting both leukocyte and platelet adhesion [101, 155]. PAR activation is also linked to the secretion of interleukins and cytokines that promote C-reactive protein synthesis that, itself, triggers many of the steps in the inflammatory process so enhancing the initiation and progression of atherosclerotic plaques [156]. Moreover, atherogenesis progressively impairs endothelial vasoactive function, ranging from impaired endothelium-dependent vasodilatation in conduit and resistance of vessels in patients with hypercholesterolemia to complete loss of endothelium-dependent vasodilation in patients with histologically proven atherosclerotic lesions [157–160].

Lipid-lowering diet [161, 162] and lifestyle modification [163, 164] have been shown to restore or improve impaired endothelium-dependent vasodilation in the atherosclerotic disease state [160].

6.2. Hypertension. Arterial hypertension is the most prevalent risk factor associated with increased cardiovascular morbidity and mortality [165]. The hypertensive vascular dysfunction, characterized by endothelial dysfunction and hypertensive remodelling of vascular smooth muscle cells, are well known and described processes. The complicated mechanisms underlying vascular dysfunction involve decreased

NO bioavailability, activation of the pathways of vascular smooth muscle contraction, vascular oxidative stress, and inflammation [166–169]. The impaired endothelium-dependent vasodilatation in hypertension is characterized by an imbalance between EC derived vasodilatant and vasoconstrictor factors [170–172]. Oxidative stress is important in the development and maintenance of hypertension, in terms of excess production of oxidants, decrease in NO bioavailability, and antioxidant capacity in the vasculature [169, 173]. Moreover, the prevalence of hypertension markedly increases with advancing aging. Although aging and hypertension, either independently or collectively, impair endothelial function, they may have similar cascades for the pathogenesis and development of endothelial dysfunction [174].

At this regard, reductions in both vascular oxidative stress and inflammation have been shown, also by our research group, that reverse endothelial dysfunction through the administration of antioxidants, such as melatonin or pycnogenol, in an experimental model of genetic hypertension [175–177].

6.3. Diabetes. Diabetes, characterized by persistent elevation of blood glucose levels (hyperglycaemia), occurs due to inadequate production of insulin (type 1 diabetes) or resistance to endogenous insulin usually associated with metabolic syndrome and obesity (type 2 diabetes).

Endothelial functions impaired in metabolic syndrome could reduce insulin access to the tissue and thus decrease insulin sensitivity independently from direct effects at the muscle cells [178]. In type 1 diabetes, endothelial dysfunction is predominantly triggered by the metabolic changes related to hyperglycemia and microvascular complications, but prevalently at retinal and kidney levels [179]. In type 2 diabetes, the link between endothelial dysfunction and diabetes is more complex, as endothelial dysfunction starts well before the onset of diabetes [180–182].

In insulin resistance and diabetes, a variety of endothelial functions is compromised, including regulation of vascular tone [182, 183] and organ perfusion [184, 185], inhibition of inflammation [182, 186], transendothelial transport of blood solutes [182, 186], prevention of coagulation [178, 185], and initiation of angiogenesis [187, 188].

Moreover, it has been shown that impaired endothelium-dependent vasorelaxation is linked to increased cav-1 protein expression in the aorta of diabetic rats. This was attributed to an inhibition of eNOS function due to cav-1 binding and a reduction of NO production [189, 190].

The endothelial insulin signalling is significantly impaired after a high fat diet, coinciding with a reduction in insulin-induced capillary recruitment and reduced interstitial insulin. Thus, it suggests that the endothelial insulin signalling required for delivery of insulin to the interstitial space can be inhibited physiologically by diet [178].

Recent studies have observed that hyperglycemia caused mitochondrial fragmentation and altered mitochondrial dynamics, associated with increase in mitochondrial ROS production [191] and so could cause a rapid breakdown in NO vascular tone regulation [58, 178]. This impairment could be responsible for the endothelial dysfunction observed in diabetes; however, endothelial dysfunction is often evident prior to a significant elevation in plasma glucose levels and can be induced by factors other than hyperglycaemia [178, 192]. In Figure 4 are schematically summarized the main pathways activated during hyperglycaemia and low insulin level inducing diabetes-associated vascular diseases.

Adipose tissue, especially in the abdomen and around blood vessels, defined perivascular adipose tissue (PVAT) [182, 193], has been shown to control insulin sensitivity and endothelial function [194], especially insulin-mediated vasoreactivity [182, 195–199]. Adipose tissue secretes a wide variety of bioactive substances (adipokines) that act directly on vascular endothelium [200]. With regard to endothelial function, PVAT is increasingly recognized as a critical fat depot that regulates local vascular tone [168] and inflammation [182, 201, 202]. These functions appear impaired in obesity and type 2 diabetes [168, 184, 203]. The PVAT inflammation may not only contribute to endothelial dysfunction but also hypoperfusion of adipose tissue and the resulting hypoxia may also trigger inflammation and alter adipokine secretion hence leading to a dysfunctional modulation of vascular tone [182, 204, 205].

6.4. Cardiac Valvular Degeneration. Cardiac valve pathology may be associated with local expression of VCAM-1 and E-selectin [5, 206, 207]. There is also evidence that aortic valve stenosis is associated with angiogenic activation of valvular ECs. In Chalajour et al.'s [208] study, normal valves were avascular, whereas stenotic aortic valves contained neovessels. ECs lining these neovessels were consistently positive for PECAM-1, but only a portion was positive for von Willebrand factor. The heart valve stimulated by myocardium signals may lead to ECs transformation in endocardial-mesenchymal cells, losing cell-cell contacts and invading the extracellular matrix so forming endocardial cushions. It is important to underline that only those ECs within this region are capable of responding to these signals [5].

Halcox and Quyyumi [209] showed that impaired endothelium-dependent vasodilatation in coronary arteries with established atherosclerosis induced vasoconstriction, which may result in reduced myocardial perfusion and myocardial ischemia. There are also evidences to suggest that abnormalities of endothelium-dependent vasodilatation contribute to the clinical syndromes of microvascular angina and coronary vasospasm [42].

6.5. Cigarette Smoking and Endothelial Dysfunction. Epidemiological studies suggest that cardiovascular diseases account for over one-third of deaths of cigarette smokers [210, 211]. Nicotine exposure via chronic cigarette smoking is an emerging cause of cardiovascular disorders [212, 213]. Numerous studies suggest that exposure to cigarette smoke leads not only to EC and vascular smooth muscle morphological alterations, but also functional exchanges [214–216] as early events in the pathogenesis of cardiovascular disease induced by cigarette smoking [217]. Nicotine plays a key role in mediating these changes by decreasing NO generation and

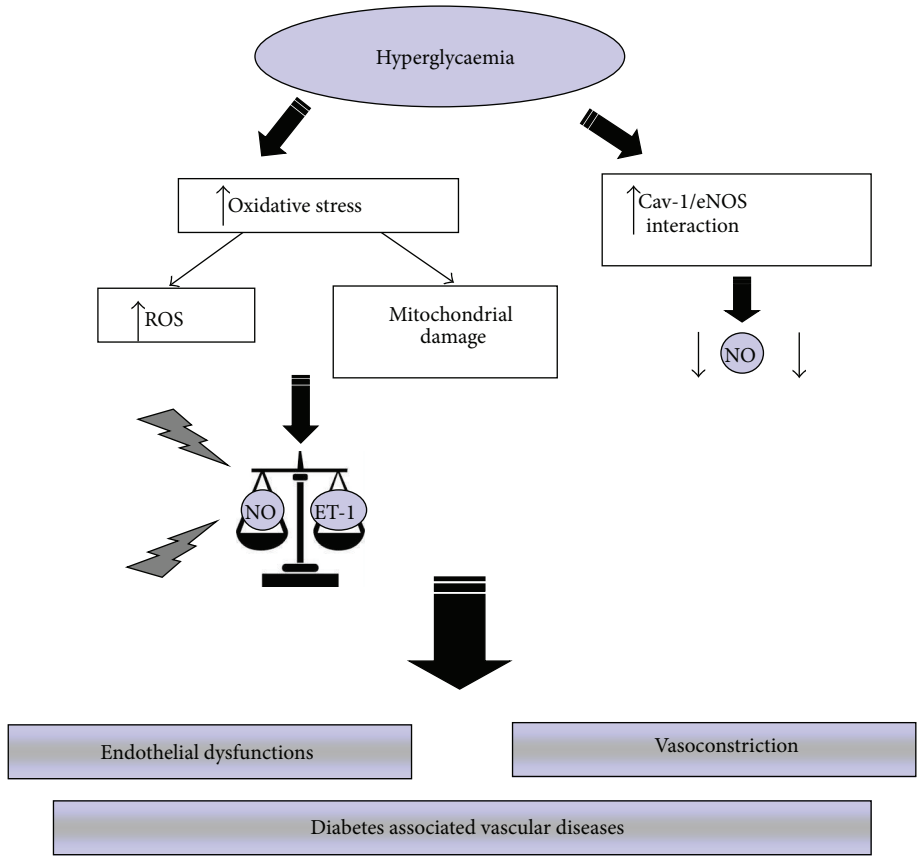

Figure 4: Main pathways activated during hyperglycaemia and low insulin level that alter the modulation of vascular tone, reducing nitric oxide, and increasing endothelin-1 release and so lead to endothelial dysfunction and diabetes-associated vascular disease. Cav-1: caveolin-1, eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; NO: nitric oxide.

bioavailability and downregulating the expression of eNOS (Figure 5) [218].

Furthermore, nicotine causes a loss of functional integrity of endothelium by causing vasospasm, stimulating the adhesion of platelets and leukocytes and promoting the formation of thrombus [219]. Our research group demonstrated that the administration of nicotine in rats depleted the bioavailability of NO, increased ROS, and damaged the structural integrity of aortic endothelium inducing cardiovascular disorders. The possible mechanism proposed to explain the damage of nicotine was the disruption of the physiological balance of vascular tone, the increased expression of ET-1, inducible NOS, and the reduced expression of eNOS and so NO and SOD [220, 221]. Furthermore, we observed both an increase in ICAM-1 and VCAM-1 expression that induced, in turn, the adhesion of monocytes and lymphocytes at EC level promoting the formation of the atherosclerotic lesion [46]. Nicotine may induce also the release of platelets derived growth factors, which promote the migration of vascular smooth muscle cells at the subendothelial space [222].

6.6. Aging and Endothelial Dysfunction. Aging is one of the main risk factors for the development of cardiovascular diseases and dysfunction at both endothelial and vascular smooth muscle cells. This dysfunction favours vasospasm, thrombosis, penetration of macrophages, cellular growth, oxidative stress, and inflammation leading to atherosclerosis and it is considered as a crucial event in the development of many vasculopathies [223]. Moreover, the aging process may deteriorate the balance between vasodilator and vasoconstriction substances produced by the endothelium [224–227]. This imbalance is mainly characterized by a progressive reduction of NO bioavailability and an increase in the production of cyclooxygenase-derived vasoconstrictor factors [223–227]. Both circumstances are in turn related to an increased production of ROS and reactive nitrogen species [223, 226]. Free radicals play a physiological role in the vessel wall; in fact, they participate as second messengers in endothelium-dependent functions, vascular smooth muscle, ECs growth and survival, and in remodelling of the vessel wall [227, 228].

The presence of endothelial dysfunction in old people is associated not only with cardiovascular diseases such as atherosclerosis, coronary artery disease, diabetes mellitus, and arterial hypertension [228] but also with diseases related with aging as renal dysfunction [229], Alzheimer's disease [223, 230], circadian cycle alterations [231], erectile dysfunction [232], osteoporosis [223, 233], and retinopathy [234]. In addition, cell senescence plays a key action in the attenuated angiogenic and regenerative capacity of ECs with aging [223].

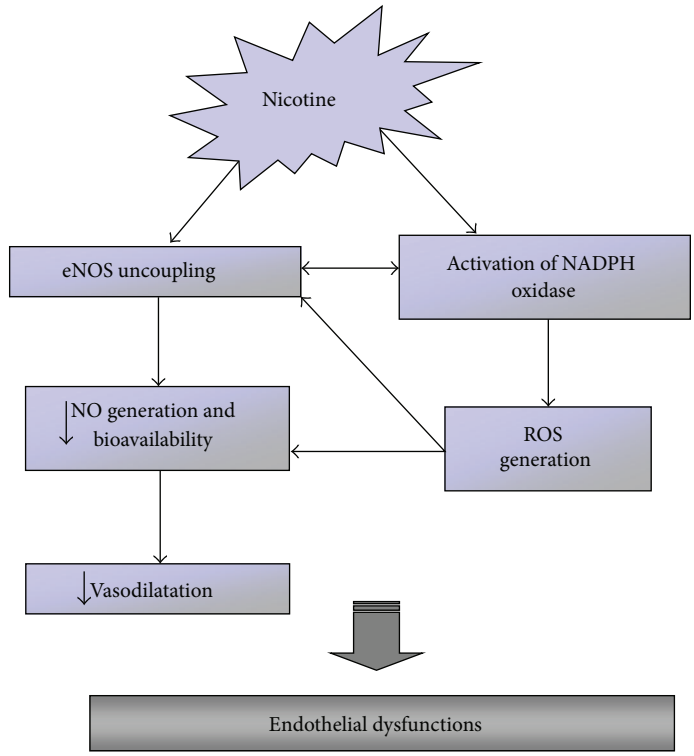

Figure 5: Schematic representation of endothelial dysfunction induced by chronic exposure to nicotine.

Senescent ECs showed a reduced proliferation that may limit the capacity to form new vascular structures. Interestingly, initial signs of endothelial senescence can even be found in young people [235, 236].

Several studies showed mitochondrial oxidative stress as a typical age-related endothelial dysfunction. This phenomenon is associated with the overactivation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme localized in the cytoplasm and in membranes of mitochondria [227, 237, 238]. Other molecular mechanisms responsible for age-related mitochondrial oxidative stress in the vasculature involve deregulation of antioxidant defences, such as peroxynitrite-mediated erythroid 2-related factor 2 (Nrf2) dysfunction, nitration and inhibition of SOD, decline in glutathione content, and a dysfunctional electron transport chain [227, 239]. Previous studies suggest that increased ROS in aging promotes mitochondrial protein oxidation and increased mitochondrial DNA mutations in heart and other organs, but it is yet to be determined whether similar aging-induced mitochondrial DNA and protein damages play an important role in ECs and vascular smooth muscle cells alterations. Furthermore, some evidences suggest that mitochondria-derived ROS contribute to accelerated development of the senescent phenotype in ECs. EC senescence may impair the physiological properties of the endothelium and may promote the progression of cardiovascular diseases by altering the secretion of cytokines, growth factors, and proteases in the vascular wall [240].

Inhibition of Sirtuin 1, a class III nicotinamide adenine dinucleotide- (NAD-) dependent protein deacetylases defined as longevity protein [47, 241, 242], induces premature senescence, whereas Sirtuin 1 overexpression reverts premature senescence induced at vascular level [227].

In our recent study, we observed that Sirtuin 1 is implicated in the development of age-related vasculopathy. Sirtuin 1 is not present in the vessels of apolipoprotein E-deficient mice at 6 weeks or 15 weeks of age. So, the studies provide a new insight into the atheroprotective effects of Sirtuin 1 and imply that Sirtuin 1 may be a potential target for the intervention in the atherogenetic process [47].

The main EC dysfunction induced by the physiopathological process of aging is summarized in Figure 6. Further studies are warranted to determine whether novel therapies that reduce mitochondrial oxidative stress and/or senescence of ECs are able to prevent the development of endothelial senescence and apoptosis, improve vasodilator functions, and prevent age vasculopathy [240].

7. Prevention of Endothelial Dysfunction

Various pharmacological therapies have been designed to reduce the development and progression of cardiovascular diseases, such as insulin sensitizers, statins, Ca^{2+} channel blockers, inhibitors of the rennin-angiotensin system, and antiplatelet agents [243–245]. However, strictly controlling cardiovascular risk factors is often difficult to obtain and the progression of pathological states has not been completely prevented with current pharmacological therapeutic options. Moreover, the modern evolution of Western societies seemingly steers populations towards a profound sedentary

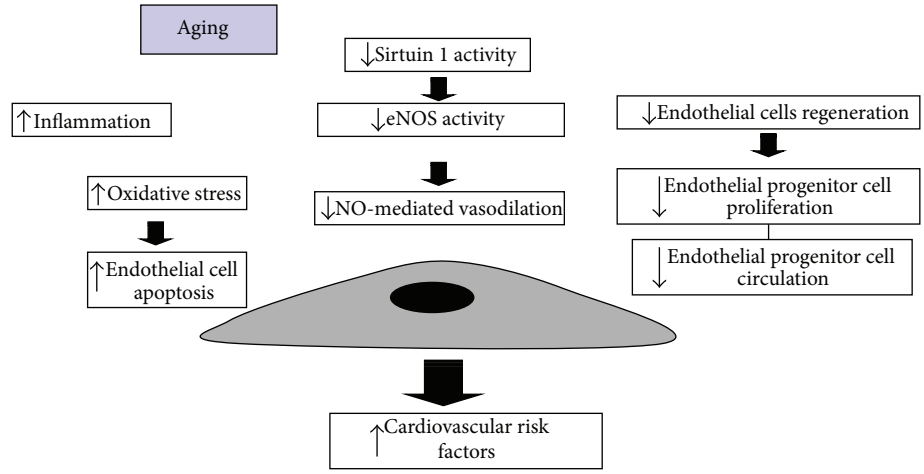

Figure 6: Schematic summary of the main age-related endothelial alterations.

lifestyle and incorrect diet is becoming difficult to reverse. Understanding the mechanisms that explain the fatal effects of physical inactivity and incorrect diet opposite to the beneficial effects of a healthy lifestyle remains largely unexplored [243, 246].

In the following paragraphs, we briefly present the beneficial effects of a healthy life style on ECs dysfunction.

7.1. Physical Activity. A large body of works has shown that moderate-intensity aerobic exercise training improves endothelial function in animals and humans with and without cardiovascular risk factors [247–251]. The multiple benefits of physical activity on survival and the harmful effects of a sedentary lifestyle made it a therapeutic modality potentially useful to treat patients with cardiovascular diseases. Its benefits regarding functional capacity can be explained by effects on endothelial properties and peripheral vascular resistance [252]. Exercise training reduces inflammation and an improvement in EC health could be a primary reason for the improvement in chronic inflammation [247].

Many studies have demonstrated evidence of abnormal endothelial function among patients with heart failure, which can be mitigated by exercise training [249]. Chronic heart failure is associated with increased levels of tumour necrosis factor-α and markers of endothelial damage, including ICAM-1 and E-selectin. Whereas acute bouts of exercise lead to an increase in proinflammatory cytokines and markers of endothelial damage, these effects were not seen when exercise was performed chronically in chronic heart failure patients [250, 251]. Ten weeks of moderate intensity exercise training improved coronary arteriolar endothelial function and reduced tumour necrosis factor-α at heart level of type 2 diabetic mice [252]. It is interesting to note that diet plus exercise training may exert remarkable benefits when either diet or exercise alone could not demonstrate so evident benefits [251].

We believe that further investigations in this exciting field would facilitate the development of physical exercise and dietary supplements as adjunctive therapies in the management of cardiovascular diseases.

7.2. Diet. Numerous studies have shown that the acute administration of a high fat diet induces a transitory disruption of endothelial function. This effect has been observed with different types of fats, including saturated, monounsaturated (MUFA), and trans fatty acids [243, 253]. It has been estimated that over 2% of total calorie intake in the developed world comes from foods containing trans fatty acids [254]. Fatty acids, produced industrially by partial hydrogenation of vegetable oils, are present in semisolid fats used in the manufacture of margarine, pastry products, frozen foods, and others. Trans fatty acids are proinflammatory factors and induce endothelial dysfunction [255, 256], which may explain the relationship between their consumption and the risk of ischemic heart disease, sudden death, and possibly diabetes [257, 258].

It has been found that Mediterranean diet, characterized by a low content of saturated fats, reduces endothelial dysfunction and markers of vascular inflammation in patients with the metabolic syndrome [259, 260], as well as reducing insulin resistance [256, 261]. de Lorgeril et al. [262] confirmed that the benefits of the Mediterranean diet, in patients recovering from myocardial infarction, are due not only to its low saturated fat and linoleic acid content, but also to the quantities of vegetables and oleic and α-linolenic acid, both found in olive oil. One possible explanation of the effects of unsaturated fatty acids is that they decrease ICAM expression, which would reduce leukocyte adhesion to dysfunctional endothelium [263]. However, in a study on a hundred patients with ischemic heart disease it was shown that a Mediterranean diet for one year did not change inflammatory or metabolic (cholesterol and triglycerides) biomarkers [256, 264]. Ong et al. [265] showed that also a diet rich in MUFA produces impairment in endothelial function when compared to a carbohydrate-rich meal. Similarly, it was observed that a diet rich in olive oil produces the same decline in flow-mediated vasodilatation as did a fast food meal [243, 253]. Lowering saturated fat intake significantly reduced atherosclerosis progression and ICAM-1 levels after two years [256, 263]. Polyunsaturated fatty acids (PUFA) appear to protect the endothelium through various mechanisms: inhibiting

expression of VCAM-1 and inducing NO synthesis [256, 266]. However, it is important to remember that slimming diets low in carbohydrates and high in protein [267] or fats [268] or low in fats and high in carbohydrates [269] may reduce weight and improve lipid profile, potentially reducing atherosclerosis and risk for coronary heart disease, but they also have significant drawbacks such as ketosis, nutritional imbalances, significant renal and liver damage, and also atherosclerotic coronary disease and obesity [256, 270, 271]. The potential atheroprotective role of Mediterranean diet during the atherogenetic process is represented in Figure 7.

Several randomized trials have shown a beneficial effect of consuming the Mediterranean diet on markers of endothelial function [258, 272, 273]. A randomized crossover study of Marin and colleagues [274] in 20 Italian elderly people evaluated the effect of a Mediterranean diet on circulating endothelial microparticles and EPCs. Activated endothelial microparticles are complex vesicular structures shed from activated or apoptotic ECs and play a remarkable role in coagulation, inflammation, and angiogenesis contributing to the progression of vascular diseases; in particular activated endothelial microparticles are released in response to the damage of the vascular endothelium, whereas EPCs are involved in the maintenance and replacement of ECs. After 2 weeks, the Mediterranean diet lowered plasma concentrations of total activated and apoptotic EMPs and increased concentrations of EPCs as compared with a high saturated fat or low-fat diet. These results suggest and confirm that the Mediterranean diet may have beneficial effects on endothelial function and so cardiovascular diseases [272].

Ingestion of high levels of saturated fat [273] appear to cause rapid alterations in endothelial function, including reduced vasodilatation. Conversely, the vasodilation induced by a low-fat high carbohydrate diet could be attributable to increased production of NO by the endothelium [256, 275]. A study of Pérez-Jiménez et al. [276] showed that the Mediterranean diet for 28 days to healthy subjects produces a decrease in plasma markers of endothelial activation, suggesting a consequent improvement in endothelial function. Similarly, the chronic consumption of low-fat diets and Mediterranean diets was observed to improve endothelial function compared to a high-fat western diet [243, 277]. Leighton et al. [278] evaluated the effects of two high fat diets: MUFA and PUFA, both of these diets contain exiguous fruits and vegetables and were administered to healthy male during 3 weeks. It was detected that both diets, independent of the type of fat, elicited a significant decline in endothelial-dependent vasodilatation. This negative effect was reverted when the subjects added two glasses of red wine per day or fruit and vegetables to their diet, suggesting that high-fat diets induce EC dysfunction that can be counteracted with the consumption of natural antioxidants so that a dietary pattern rich in fruit, vegetables, fish, and olive oil appears to have beneficial effects on endothelial functions.

7.2.1. Polyphenols. A large number of studies have investigated the role of dietary components such as polyphenols and antioxidants, proposing that diet may prevent endothelial dysfunction hence improving endothelial function [272]. Polyphenols are the most abundant antioxidants in human diet and are widespread constituents of fruits, vegetables, cereals, olive, legumes, chocolate, and beverages, such as tea, coffee, and wine [279, 280]. It is important to underline that many dietary polyphenols possess both direct and indirect antioxidant activities. The term direct and indirect antioxidants was originally used by the Food and Drug Administration to distinguish nutrients that "*trap and deactivate reactive oxygen molecules*" (e.g., vitamin C, vitamin E, β-carotene) from those that are "*precursors of coenzymes that are involved in oxidative stress but do not have direct antioxidant activities*" (e.g., zinc, selenium, riboflavin). Later, these terms were redefined by Dinkova-Kostova and Talalay [281] who described two types of small-molecule antioxidants that protect against cellular oxidative damage [282, 283]. Various studies indicate that polyphenols may induce the upregulation of endogenous antioxidant enzymes and cytoprotective proteins such as quinone oxidoreductase-1, SOD, glutathione S-transferase, glutathione peroxidase, and heme oxygenase-1 hence exerting also an indirect antioxidant effect in addition to direct radical scavenging [283, 284].

Despite their wide distribution, the health effects of dietary polyphenols have been attentively studied only in recent years and several studies, although not all, have found an inverse association between polyphenol consumption and cardiovascular disease mortality [285].

Moreover, flavonoids, many of which are polyphenolic compounds, are believed to be beneficial for the prevention and treatment of cardiovascular diseases mainly by decreasing oxidative stress and increasing vasodilatation [279, 286, 287]. More than 8.000 different flavonoids have been described and since they are prerogative of the kingdom of plants, they are part of human diet with a daily total intake amounting to 1 g, which is higher than all other classes of phytochemicals and known dietary antioxidants. In fact, the daily intake of vitamin C, vitamin E, and β-carotene from food is estimated minor of 100 mg [279]. A large number of human intervention studies have evaluated the beneficial effects of flavonoid intake and foods containing flavonoids on endothelial function. Morand et al. [288] investigated the acute and 4-week effects of orange juice and its major flavonoid, hesperidin, on microvascular reactivity in a crossover study in 24 healthy overweight men and observed that the intake of orange juice flavonoids may have acute beneficial effects on endothelium but it does not provide evidence for a long-term administration. Moreover, epidemiological studies have suggested that regular moderate consumption of red wine, rich in flavonoids, is associated with increased NO expression hence the positive modulation of vascular tone and that consumption of cacao and green tea improved endothelial functions and this effect appears to be at least partly mediated by flavonoid components, like catechins.

Similarly, another study suggested that short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary heart disease [243, 289].

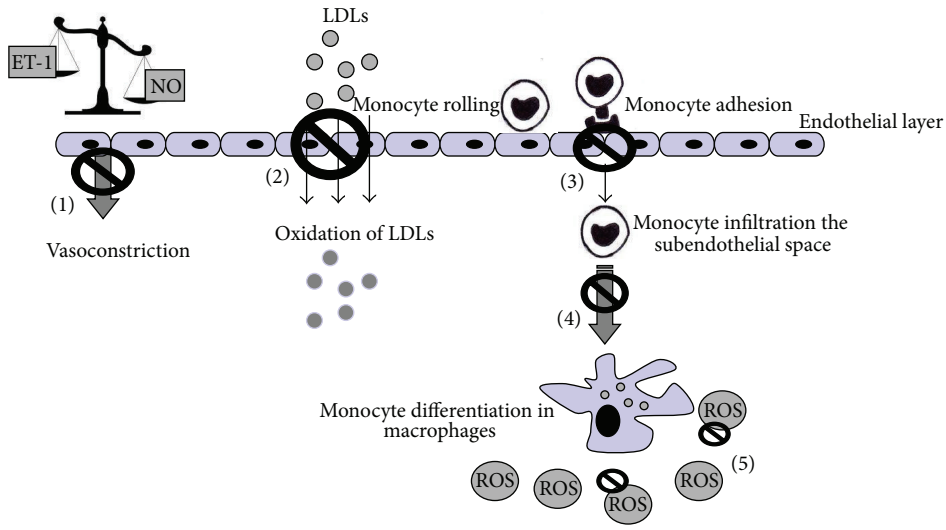

Figure 7: Potential atheroprotective role of Mediterranean diet during the atherogenetic process. The Mediterranean diet, with mainly its antioxidant properties, may (1) inhibit/reduce the imbalance between vasoconstrictors and vasodilators, (2) block oxidation of low density lipoproteins, (3) inhibit monocytes adhesion to endothelial cells constraining monocytes infiltration in the subendothelial space, (4) repress differentiation of macrophages in lipid-laden macrophages, defined as foam cells, and (5) reduce radical oxygen species syntheses. ET-1: endothelin-1; LDLs: low density lipoproteins; NO: nitric oxide; ROS: radical oxygen species.

For other foods such as citrus fruit and apples, the evidence for beneficial effects on endothelial function is currently under consideration.

The main problem in studing the beneficial effects of foods is judging whether the effect comes from specific compounds or from other bioactive compounds of these foods. Only a few intervention studies have been conducted with pure compounds, so it is important to take into account the possible synergistic effects of multiple dietary components that are part of a dietary pattern and so simulate better the reality of human lifestyle.

In the following paragraphs, we briefly describe the beneficial effects on the endothelium of two of the main food components of the Mediterranean diet, olive oil and red wine, and of some of the principal antioxidant supplementation actually studied [243].

7.2.2. Olive Oil. Olive oil is the main fat in the Mediterranean diet. The wide range of antiatherogenic effects associated with olive oil consumption could contribute to explain the low rate of cardiovascular mortality found in southern European Mediterranean countries, in comparison with other western countries, despite a high prevalence of coronary heart disease factors [290]. Olive oil, particularly a virgin olive oil rich diet, decreases prothrombotic environment, modifies platelet adhesion, coagulation, and fibrinolysis, and may improve endothelial function [290, 291]. The healthful properties of olive oil have been often attributed to its high content of MUFA, namely, oleic acid [292], but it is becoming clear that the content of oleic acid alone cannot fully explain the impact of olive oil on health. It should be underlined that olive oil, unlike other vegetable oils, contains high amounts of several micronutrient constituents, including polyphenolic compounds (100–1000 mg/kg) [293]. Around 80% or more of the olive oil phenolic compounds are lost in the refination process; thus, their content is higher in virgin olive oil than in other olive oils.

The cardioprotective effects of olive oil have been ascribed to its content of MUFA and the presence of other biologically minor constituents like polyphenols, tocopherols, and triterpenoids [260, 294, 295]. Among these compounds, tocopherols and phenolic compounds have demonstrated antioxidant properties that may improve endothelial function by reducing levels of ROS in the endothelium and, consequently, the production of endothelial adhesion molecules. Moreover, extra virgin olive oil through its phenolic compounds may increase glutathione reductase and glutathione peroxidase activities [296, 297].

At this regard, some findings suggest that both major and minor olive oil components may modulate inflammation and endothelial activation. In cultured ECs, oleic acid inhibited the expression of VCAM-1 mRNA levels, monocyte adhesion, and NF-κB [298, 299]. In animal models, a diet rich in olive oil suppressed natural killer cell activity and the expression of receptors for interleukins [300, 301]. Isolated human LDL enriched in oleic acid reduced monocyte chemotaxis and adhesion, compared with linoleic-enriched LDL when being exposed to oxidative stress [290, 302]. Phytosterols and triterpenoids have anti-inflammatory and vasodilatation effects, respectively, but their roles in the endothelium need to be further studied. The increasing investigations on the properties of these minor compounds of olive oil may help to explain not only some of the classic beneficial effects of the Mediterranean diet, but also the emergence of other olive-derived oils, such as pomace olive oil, which, being enriched with these minor components, might be helpful in preventing cardiovascular diseases [291].

Olive oil rich Mediterranean diet has been observed to improve the endothelium-dependent dilatation in hypercholesterolemic males [277, 290] and metabolic syndrome patients [261]. Ryan et al. [261] showed that an olive oil diet attenuates the endothelial dysfunction present during the consumption of a baseline diet high in PUFA. Moreover, Ruano et al. [303] reported that a meal containing high-phenolic virgin olive oil improves the endothelial-dependent vasodilatation during postprandial state more than when the meal was taken with a similar olive oil, but with low-phenolic content.

Olive oil minor components have also been involved in the antioxidant activity of olive oil. Some components of the unsaponifiable fraction, such as squalene, β-sitosterol or triterpenes, have been shown to display antioxidant and chemopreventive activities and capacity to improve endothelial function decreasing the expression of cell adhesion molecules and increasing vasorelaxation [304].

The mechanisms by which olive oil and its components exert beneficial effects merit further investigation and further studies are required to obtain evidence of the benefits of olive oil consumption on primary end points for cardiovascular disease.

7.2.3. Red Wine. Wine has been part of the human culture for more than 3000 years, serving dietary and socioreligious functions [305]. Several prospective studies have consistently demonstrated that weak/moderate red wine consumption (one to two drinks per day) is strongly associated with a lower incidence of cardiovascular diseases compared with teetotal or occasional alcohol consumption. The cardiovascular benefits of wine are likely due to combined, additive, or perhaps synergistic effects of alcohol and other wine components (mainly resveratrol and other polyphenolic compounds) on atherogenesis, coagulation, and fibrinolysis [305, 306]. Improved vascular function was shown in several experimental models of human diseases after administration of either red wine or its constituents and it was observed that the mechanisms involved in beneficial effects of natural polyphenols include also a reduction of platelet aggregation [307], increased high density lipoprotein (HDL) concentration [308], reduced LDL oxidation and concentration [307, 309, 310], and improvement of antioxidant defence system [311, 312]. Besides antioxidant properties, which play a significant role in cardioprotection, red wine polyphenols affect vascular function via modulation of NO bioavailability [313, 314]. Red wine compounds prevented metabolic and cardiovascular alterations in obese rats [310], improved endothelial-mediated dilatation and plasma NO level in hypercholesterolemic rabbits [307, 315], and restored endothelial function in deoxycorticosterone acetate-salt hypertensive rats [316]. In rats with fully developed NO-deficient hypertension, administration of red wine compounds produced a greater blood pressure decrease and improved endothelial functions [307].

Resveratrol, natural polyphenol found in grapes and grape products, including wine, as well as other sources [317], also decreased the gene expression of the potent vasoconstrictor ET-1 [305, 318]. Corder et al. [319] showed that red wine is capable of reducing ET-1 synthesis leading again to enhanced vasodilation. Our research group observed that treatment with resveratrol of type I diabetic rats may induce increase in adiponectin, the main adipokine secreted by fat deposits, that is strictly associated with a significant decrease in circulating ECs and EC fragmentations and a significant increase in PECAM-1 positive cells; moreover, we observed that resveratrol treatment may decrease ICAM-1 and VCAM-1 and caspase-3 activity in ECs, while increasing eNOS activity [320].

In humans, red wine and red wine compounds reduced blood pressure and improved forearm endothelial functions and NO production in young healthy individuals [321]. Similarly, a decrease of blood pressure in association with elevation of plasma NO was observed after both red wine and red wine compounds consumption in men at high cardiovascular risk [307, 322]. Additionally, treatment with red wine was found to reduce the susceptibility of LDLs to oxidation and to improve endothelium-dependent vasodilatation in patients with coronary heart disease [307, 323]. On the other hand, Andrade et al. [324] observed increased endothelial-mediated dilatation after short-term red wine consumption only in hypercholesterolemic, but not in hypertensive or healthy subjects [307]. However, in a study comparing the effect of water, red wine, beer, and vodka in healthy young subjects Huang et al. [325] found that only red wine affected endothelial function and significantly increased plasma levels of NO. Moreover, it is important to remember that, regarding the biological mechanisms linking endothelial function to moderate wine intake, it has been reported that alcohol *per se* can induce a marked increase in NO synthesis in primary cultures of bovine aortic ECs and human umbilical ECs, sustained by a rapid increase of eNOS protein and mRNA expression levels. In particular, both eNOS protein and mRNA increase by nearly twofold within 3 hours and gradually decline after 0.1% ethanol ingestion, but the increased levels of mRNA persist up to 24 hours [305, 326]. A 20-hour treatment of human umbilical vein ECs with an alcohol-free red wine polyphenol extract also led to a concentration-dependent increase in NO release, associated with an up to twofold increase of human eNOS promoter activity. Remarkably, although polyphenol extracts from wines of specific origin and grape cultivars vary strongly in their individual activity, when averaged, the activity cannot be attributed to a specific grape cultivar or growing area [305, 327]. Cuevas and colleagues [328] observed that moderate red wine consumption counteracts endothelial dysfunction induced by a high fat western diet administered to healthy men. The effects of ethanol and polyphenols on NO and consequently on endothelial function are very important, since endothelial function is an early indicator of atherosclerosis and vessel damage [316, 329, 330]. Moderate red wine consumption improved neovascularization and blood flow recovery after ischemia in hypercholesterolemic mice and had a positive effect on EPCs number and functional activity [331, 332]. Huang et al. [325] reported that red wine consumption by healthy subjects enhanced circulating EPC levels and improved EPC

functions by modifying NO bioavailability. These studies support a modulatory effect of ethanol and/or polyphenols on EPC that may be antiatherogenic. Moreover, in a single blind crossover study, Whelan et al. [333] reported that the consumption of 4 mL/kg of either red or white wine with a light meal acutely improved brachial endothelial-mediated dilatation after 6 hours, whereas blood-alcohol levels had returned to baseline values by this time; the magnitude of this effect on endothelial function was substantially similar after both red and white wines. In healthy subjects, ingestion of 250 to 500 mL of red wine or dealcoholized red wine increased, in some, but not all studies, flow-mediated vasodilatation as assessed in the brachial artery by plethysmography [334–336].

It is still unclear whether the beneficial effects of red wine intake can be attributed to any specific type of grape and therefore any single wine source cannot be considered better than any other and it remains to be determined whether the reported benefits of red wine are based on one or more socioeconomic confounders. Accordingly, the beneficial effects of red wine intake in human health should be better defined and additional research works are required before any firm recommendation can be made to abstainers to initiate a light to moderate consumption of red wine [305]. Therefore, until more studies are conducted in order to elucidate this matter, it is not safe to advise consumption of a glass of red wine, as it is not clear if it would benefit or harm endothelial function in the immediate postprandial state [314].

7.2.4. Antioxidants Supplementation

Tempol. Due to the central role of oxidative stress in development of cardiovascular diseases, many studies have been conducted over the past 20 years to investigate the potential use of antioxidants or mimetic of endogenous antioxidants, such as vitamins C and E, to reduce cardiovascular alterations [272].

Administration of tempol, a cell permeable SOD mimetic, has shown improvements in diabetes associated microvascular complications, such as nephropathy and retinopathy [59, 337]. In addition, tempol restores endothelial vasodilatation in large conduit vessels of alloxan-induced diabetic rabbits [58, 336]. Tempol increases endothelium-dependent vasodilatation in arteries from hypertensive animals, most likely through a lowering of ROS, but other mechanisms also appear to contribute to the effect [295].

MN40403, another highly specific nonpeptide SOD mimetic, was able to reverse endothelial dysfunction *ex vivo* by targeting NADPH oxidase-mediated superoxide production at aorta level of apolipoprotein E-deficient mice [58, 338].

Vitamins C and E. It has been detected that vitamins C and vitamin E, water, and lipid soluble antioxidants, respectively, produce beneficial effects on endothelial functions by modulating the downregulation of eNOS expression [339]. Moreover, the deleterious effects of postprandial hypertriglyceridemia on endothelial-dependent vasodilation can be counteracted by the simultaneous administration of vitamins C and E [243, 340, 341]. Maio et al. [342] investigated the acute effects of arterial vitamin C infusion on forearm blood flow in response to acetylcholine (a NO-dependent vasodilator) in 190 dipper and nondipper hypertensive patients (in dipper patients, but not in nondipper patients, blood pressure drops during night period). The authors found that vitamin C treatment improved blood flow following acetylcholine administration in both nondipper and dipper patients, but particularly in dipper ones. This observation supports and confirms the hypothesis that vitamin C improves endothelial function in hypertensive patients with impaired NO physiology. Vitamin C intake has also been shown to increase muscular blood flow during exercise in older adults and this effect is associated with improved endothelium-dependent vasodilatation mainly due to increased NO availability via eNOS [272, 343, 344].

The antioxidant effects of vitamins C and E are well established. However, studies both *in vitro* [345, 346] and *in vivo* [347, 348] have shown that antioxidant vitamins, particularly vitamin E, can have a paradoxical prooxidant effect when administrated under conditions of normal or low basal oxidative stress [349]. Indeed, the general trend towards increased cardiovascular mortality has opened a serious debate about the safety of vitamin E supplementation in high risk individuals. Further evidence about the efficacy of antioxidant vitamins is awaited from other ongoing trials of vitamin E alone or in combination with other antioxidants. Trying to explain the failure of vitamin E, it has been suggested that antioxidant therapy may require a longer treatment period because its primary mechanism of action is the prevention of new lesion formation [350].

Melatonin. Melatonin, an endogenously produced indoleamine, is a remarkably functionally pleiotropic molecule [351] which functions as a highly effective antioxidant and free radical scavenger [352, 353]. Endogenously produced and exogenously administered melatonin has known beneficial actions on the cardiovascular system [354, 355]. In particular, the antioxidant action of melatonin and its possible interaction with EDHF may contribute to blood pressure lowering effect, which was observed even when the NO pathway was inhibited [356]. However, melatonin seems to increase NO levels either through the stimulation of NO production and/or the prevention of coupling to the superoxide anion radical [357]. In addition melatonin was able to modulate *in vitro* acetylcholine-induced relaxation or phenylephrine-induced vasoconstriction of aortic rings of aging rats [358, 359] and New Zealand rabbits, respectively, in an endothelium-dependent manner [360]. Nevertheless, the mechanisms of these antihypertensive effects of melatonin are actually not completely understood.

Melatonin also may inhibit endothelium-derived adhesion molecules formation, reduce fatty acids infiltration in the intimal layer [361], and neutralize free radicals [362, 363]. Moreover, melatonin was demonstrated to prevent tissue injury and structural and functional alterations in the vasculature induced by cigarette smoking. Our research group observed and confirmed that melatonin minimized

the damage induced by nicotine, reestablished the physiological balance between vasodilatation (increasing eNOS) and vasoconstriction (decreasing ET-1), induced antioxidant enzymes, and downregulated adhesive molecules on ECs [46, 220, 221].

8. Conclusions

Actually, compelling evidences indicate that an increased consumption of correct diet containing nutritive and nonnutritive compounds may contribute to the improvement of the quality of life by delaying onset and reducing the risk of cardiovascular diseases and, in particular, the development of endothelial dysfunction. In this context, wine, tea, fruits, vegetables, and olive oil received much attention, because they are particularly rich in natural antioxidants.

However, a better understanding of the mechanism(s) underlying EC dysfunction during cardiovascular physiopathology is a prerequisite for effective pharmacological and nonpharmacological interventions and treatments.

In conclusion, the proposal that antioxidants may ameliorate endothelial dysfunction is very interesting and promising, but further studies are needed to better understand the mechanisms that underline the biological effect of healthy life style.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Rita Rezzani and Luigi Fabrizio Rodella equally contributed to this paper.

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References

[1] T. Gori, S. Dragoni, G. di Stolfo, and S. Forconi, "Endothelium and haemorheology," *Annali dell'Istituto Superiore di Sanita*, vol. 43, no. 2, pp. 124–129, 2007.

[2] T. Minami and W. C. Aird, "Endothelial cell gene regulation," *Trends in Cardiovascular Medicine*, vol. 15, no. 5, pp. 174–184, 2005.

[3] W. C. Aird, "Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms," *Circulation Research*, vol. 100, no. 2, pp. 158–173, 2007.

[4] E. R. Weibel and G. E. Palade, "New cytoplasmic components in arterial endothelia," *The Journal of Cell Biology*, vol. 23, pp. 101–112, 1964.

[5] W. C. Aird, "Phenotypic heterogeneity of the endothelium: II. Representative vascular beds," *Circulation Research*, vol. 100, no. 2, pp. 174–190, 2007.

[6] K. Yano, D. Gale, S. Massberg et al., "Phenotypic heterogeneity is an evolutionarily conserved feature of the endothelium," *Blood*, vol. 109, no. 2, pp. 613–615, 2007.

[7] W. C. Aird, "Endothelium in health and disease," *Pharmacological Reports*, vol. 60, no. 1, pp. 139–143, 2008.

[8] H. F. Galley and N. R. Webster, "Physiology of the endothelium," *British Journal of Anaesthesia*, vol. 93, no. 1, pp. 105–113, 2004.

[9] H. G. Augustin, D. H. Kozian, and R. C. Johnson, "Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes," *BioEssays*, vol. 16, no. 12, pp. 901–906, 1994.

[10] D. B. Cines, E. S. Pollak, C. A. Buck et al., "Endothelial cells in physiology and in the pathophysiology of vascular disorders," *Blood*, vol. 91, no. 10, pp. 3527–3561, 1998.

[11] J.-P. Girard and T. A. Springer, "High Endothelial Venules (HEVs): specialized endothelium for lymphocyte migration," *Immunology Today*, vol. 16, no. 9, pp. 449–457, 1995.

[12] M. Miyasaka and T. Tanaka, "Lymphocyte trafficking across high endothelial venules: dogmas and enigmas," *Nature Reviews Immunology*, vol. 4, no. 5, pp. 360–370, 2004.

[13] F. Florey, "The endothelial cell," *British Medical Journal*, vol. 2, no. 5512, pp. 487–490, 1966.

[14] E. Wisse, "An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids," *Journal of Ultrasructure Research*, vol. 31, no. 1-2, pp. 125–150, 1970.

[15] M. Simionescu, N. Simionescu, and G. E. Palade, "Morphometric data on the endothelium of blood capillaries," *The Journal of Cell Biology*, vol. 60, no. 1, pp. 128–152, 1974.

[16] A. Caggiati, M. Phillips, A. Lametschwandtner, and C. Allegra, "Valves in small veins and venules," *European Journal of Vascular and Endovascular Surgery*, vol. 32, no. 4, pp. 447–452, 2006.

[17] D. Feng, J. A. Nagy, H. F. Dvorak, and A. M. Dvorak, "Ultrastructural studies define soluble macromolecular, particulate, and cellular transendothelial cell pathways in venules, lymphatic vessels, and tumor-associated microvessels in man and animals," *Microscopy Research and Technique*, vol. 57, no. 5, pp. 289–326, 2002.

[18] P. S. Frenette, R. C. Johnson, R. O. Hynes, and D. D. Wagner, "Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 16, pp. 7450–7454, 1995.

[19] M. Mori, J. W. Salter, T. Vowinkel, C. F. Krieglstein, K. Y. Stokes, and D. N. Granger, "Molecular determinants of the prothrombogenic phenotype assumed by inflamed colonic venules," *American Journal of Physiology*, vol. 288, no. 5, pp. G920–G926, 2005.

[20] A. Tailor and D. N. Granger, "Hypercholesterolemia promotes leukocyte-dependent platelet adhesion in murine postcapillary venules," *Microcirculation*, vol. 11, no. 7, pp. 597–603, 2004.

[21] D. Cooper, J. Russell, K. D. Chitman, M. C. Williams, R. E. Wolf, and D. N. Granger, "Leukocyte dependence of platelet adhesion in postcapillary venules," *American Journal of Physiology*, vol. 286, no. 5, pp. H1895–H1900, 2004.

[22] G. Kraal and R. E. Mebius, "High endothelial venules: lymphocyte traffic control and controlled traffic," *Advances in Immunology*, vol. 65, pp. 347–395, 1997.

[23] U. H. von Andrian and T. R. Mempel, "Homing and cellular traffic in lymph nodes," *Nature Reviews Immunology*, vol. 3, no. 11, pp. 867–878, 2003.

[24] P. R. Streeter, B. T. Rouse, and E. C. Butcher, "Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes," *The Journal of Cell Biology*, vol. 107, no. 5, pp. 1853–1862, 1988.

[25] A. O. Anderson and S. Shaw, "T cell adhesion to endothelium: the FRC conduit system and other anatomic and molecular features which facilitate the adhesion cascade in lymph node," *Seminars in Immunology*, vol. 5, no. 4, pp. 271–282, 1993.

[26] D. L. Brutsaert, "Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity," *Physiological Reviews*, vol. 83, no. 1, pp. 59–115, 2003.

[27] D. A. Narmoneva, R. Vukmirovic, M. E. Davis, R. D. Kamm, and R. T. Lee, "Endothelial cells promote cardiac myocyte survival and spatial reorganization: implications for cardiac regeneration," *Circulation*, vol. 110, no. 8, pp. 962–968, 2004.

[28] K. Yamamoto, V. de Waard, C. Fearns, and D. J. Loskutoff, "Tissue distribution and regulation of murine von Willebrand factor gene expression in vivo," *Blood*, vol. 92, no. 8, pp. 2791–2801, 1998.

[29] H. Cai, Z. Li, A. Goette et al., "Downregulation of endocardial nitric oxide synthase expression and nitric oxide production in atrial fibrillation: potential mechanisms for atrial thrombosis and stroke," *Circulation*, vol. 106, no. 22, pp. 2854–2858, 2002.

[30] L. J. Andries, D. L. Brutsaert, and S. U. Sys, "Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium," *Circulation Research*, vol. 82, no. 2, pp. 195–203, 1998.

[31] J. Bischoff and E. Aikawa, "Progenitor cells confer plasticity to cardiac valve endothelium," *Journal of Cardiovascular Translational Research*, vol. 4, no. 6, pp. 710–719, 2011.

[32] R. J. Esper, R. A. Nordaby, J. O. Vilariño, A. Paragano, J. L. Cacharrón, and R. A. Machado, "Endothelial dysfunction: a comprehensive appraisal," *Cardiovascular Diabetology*, vol. 5, article 4, 2006.

[33] E. Dejana, M. Corada, and M. G. Lampugnani, "Endothelial cell-to-cell junctions," *FASEB Journal*, vol. 9, no. 10, pp. 910–918, 1995.

[34] K. A. Pasyk and B. A. Jakobczak, "Vascular endothelium: recent advances," *European Journal of Dermatology*, vol. 14, no. 4, pp. 209–213, 2004.

[35] A. G. Passerini, D. C. Polacek, C. Shi et al., "Coexisting proinflammatory and antioxidative endothelial transcription profiles in a disturbed flow region of the adult porcine aorta," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 8, pp. 2482–2487, 2004.

[36] C. Lupu, A. D. Westmuckett, G. Peer et al., "Tissue factor-dependent coagulation is preferentially up-regulated within arterial branching areas in a baboon model of *Escherichia coli* sepsis," *American Journal of Pathology*, vol. 167, no. 4, pp. 1161–1172, 2005.

[37] M. P. Bevilacqua, J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone Jr., "Identification of an inducible endothelial-leukocyte adhesion molecule," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 24, pp. 9238–9242, 1987.

[38] M. P. Bevilacqua, S. Stengelin, M. A. Gimbrone Jr., and B. Seed, "Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins," *Science*, vol. 243, no. 4895, pp. 1160–1165, 1989.

[39] M. Uematsu, Y. Ohara, J. P. Navas et al., "Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress," *American Journal of Physiology*, vol. 269, no. 6, part 1, pp. C1371–C1378, 1995.

[40] S. Verma, M. R. Buchanan, and T. J. Anderson, "Endothelial function testing as a biomarker of vascular disease," *Circulation*, vol. 108, no. 17, pp. 2054–2059, 2003.

[41] M. Yanagisawa, A. Inoue, T. Ishikawa et al., "Primary structure, synthesis, and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 18, pp. 6964–6967, 1988.

[42] J. W. Butany, S. Verma, R. L. Leask, B. Mohsen, and S. L. Asa, "Genetic abnormalities of the endothelium," *Microscopy Research and Technique*, vol. 60, no. 1, pp. 30–37, 2003.

[43] E. Thorin and D. J. Webb, "Endothelium-derived endothelin-1," *Pflugers Archiv*, vol. 459, no. 6, pp. 951–958, 2010.

[44] R. Rezzani, L. Rodella, and R. Bianchi, "Induction of endothelin in rat kidney after cyclosporine A treatment," *Acta Histochemica*, vol. 103, no. 4, pp. 423–431, 2001.

[45] R. Rezzani, L. Rodella, B. Buffoli et al., "Change in renal heme oxygenase expression in cyclosporine A-induced injury," *Journal of Histochemistry and Cytochemistry*, vol. 53, no. 1, pp. 105–112, 2005.

[46] L. F. Rodella, F. Filippini, F. Bonomini, R. Bresciani, R. J. Reiter, and R. Rezzani, "Beneficial effects of melatonin on nicotine-induced vasculopathy," *Journal of Pineal Research*, vol. 48, no. 2, pp. 126–132, 2010.

[47] L. F. Rodella, G. Favero, C. Rossini et al., "Aging and vascular dysfunction: beneficial melatonin effects," *Age*, vol. 35, no. 1, pp. 103–115, 2013.

[48] M.-E. Lee, K. D. Bloch, J. A. Clifford, and T. Quertermous, "Functional analysis of the endothelin-1 gene promoter. Evidence for an endothelial cell-specific cis-acting sequence," *Journal of Biological Chemistry*, vol. 265, no. 18, pp. 10446–10450, 1990.

[49] G. P. Rossi, A. Sacchetto, M. Cesari, and A. C. Pessina, "Interactions between endothelin-1 and the renin-angiotensin-aldosterone system," *Cardiovascular Research*, vol. 43, no. 2, pp. 300–307, 1999.

[50] G. P. Rossi, A. Sacchetto, D. Rizzoni et al., "Blockade of angiotensin II type 1 receptor and not of endothelin receptor prevents hypertension and cardiovascular disease in transgenic (mREN2)27 rats via adrenocortical steroid-independent mechanisms," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 4, pp. 949–956, 2000.

[51] A. W. Hahn, T. J. Resink, T. Scott-Burden, J. Powell, Y. Dohi, and F. R. Bühler, "Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: a novel autocrine function," *Cell Regulation*, vol. 1, no. 9, pp. 649–659, 1990.

[52] C.-P. Sung, A. J. Arleth, B. L. Storer, and E. H. Ohlstein, "Angiotensin type 1 receptors mediate smooth muscle proliferation and endothelin biosynthesis in rat vascular smooth muscle," *Journal of Pharmacology and Experimental Therapeutics*, vol. 271, no. 1, pp. 429–437, 1994.

[53] H. Ito, Y. Hirata, S. Adachi et al., "Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes," *Journal of Clinical Investigation*, vol. 92, no. 1, pp. 398–403, 1993.

[54] P. Moreau, H. Takase, C. F. Küng, S. Shaw, and T. F. Lüscher, "Blood pressure and vascular effects of endothelin blockade in chronic nitric oxide-deficient hypertension," *Hypertension*, vol. 29, no. 3, pp. 763–769, 1997.

[55] R. P. McEver, J. H. Beckstead, K. L. Moore, L. Marshall-Carlson, and D. F. Bainton, "GMP-140, a platelet α-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies," *Journal of Clinical Investigation*, vol. 84, no. 1, pp. 92–99, 1989.

[56] K. Iiyama, L. Hajra, M. Iiyama et al., "Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation," *Circulation Research*, vol. 85, no. 2, pp. 199–207, 1999.

[57] M. D. Chávez, N. Lakshmanan, and M. Kavdia, "Impact of superoxide dismutase on nitric oxide and peroxynitrite levels in the microcirculation—a computational model," in *Proceedings of the 29th Annual International Conference of IEEE Engineering in Medicine and Biology Society (EMBS '07)*, pp. 1022–1026, Lyon, France, August 2007.

[58] A. Sharma, P. N. Bernatchez, and J. B. de Haan, "Targeting endothelial dysfunction in vascular complications associated with diabetes," *International Journal of Vascular Medicine*, vol. 2012, Article ID 750126, 12 pages, 2012.

[59] C.-L. M. Cooke and S. T. Davidge, "Endothelial-dependent vasodilation is reduced in mesenteric arteries from superoxide dismutase knockout mice," *Cardiovascular Research*, vol. 60, no. 3, pp. 635–642, 2003.

[60] H. Goto, T. Nishikawa, K. Sonoda et al., "Endothelial MnSOD overexpression prevents retinal VEGF expression in diabetic mice," *Biochemical and Biophysical Research Communications*, vol. 366, no. 3, pp. 814–820, 2008.

[61] J. Contrino, G. Hair, D. L. Kreutzer, and F. R. Rickles, "In situ detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease," *Nature Medicine*, vol. 2, no. 2, pp. 209–215, 1996.

[62] J. Crawley, F. Lupu, A. D. Westmuckett, N. J. Severs, V. V. Kakkar, and C. Lupu, "Expression, localization, and activity of tissue factor pathway inhibitor in normal and athrosclerotic human vessels," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 5, pp. 1362–1373, 2000.

[63] A. Solovey, R. Kollander, A. Shet et al., "Endothelial cell expression of tissue factor in sickle mice is augmented by hypoxia/reoxygenation and inhibited by lovastatin," *Blood*, vol. 104, no. 3, pp. 840–846, 2004.

[64] T. Nagayasu, S. Saadi, R. A. Holzknecht, T. B. Plummer, and J. L. Platt, "Expression of tissue factor mRNA in cardiac xenografts: clues to the pathogenesis of acute vascular rejection," *Transplantation*, vol. 69, no. 4, pp. 475–482, 2000.

[65] E. Dejana, "Endothelial cell-cell junctions: happy together," *Nature Reviews Molecular Cell Biology*, vol. 5, no. 4, pp. 261–270, 2004.

[66] G. Bazzoni and E. Dejana, "Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis," *Physiological Reviews*, vol. 84, no. 3, pp. 869–901, 2004.

[67] M. G. Farquhar and G. E. Palade, "Junctional complexes in various epithelia," *The Journal of Cell Biology*, vol. 17, pp. 375–412, 1963.

[68] G. van Meer, B. Gumbiner, and K. Simons, "The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next," *Nature*, vol. 322, no. 6080, pp. 639–641, 1986.

[69] E. A. Runkle and D. Mu, "Tight junction proteins: from barrier to tumorigenesis," *Cancer Letters*, vol. 337, no. 1, pp. 41–48, 2013.

[70] G. Bazzoni, "The JAM family of junctional adhesion molecules," *Current Opinion in Cell Biology*, vol. 15, no. 5, pp. 525–530, 2003.

[71] K. Ebnet, "Organization of multiprotein complexes at cell-cell junctions," *Histochemistry and Cell Biology*, vol. 130, no. 1, pp. 1–20, 2008.

[72] J. D. Schulzke, D. Günzel, L. J. John, and M. Fromm, "Perspectives on tight junction research," *Annals of the New York Academy of Sciences*, vol. 1257, pp. 1–19, 2012.

[73] F. R. Curry and R. H. Adamson, "Tonic regulation of vascular permeability," *Acta Physiologica*, vol. 207, no. 4, pp. 628–649, 2013.

[74] E. Dejana and C. Giampietro, "Vascular endothelial-cadherin and vascular stability," *Current Opinion in Hematology*, vol. 19, no. 3, pp. 218–223, 2012.

[75] H. Drexler, A. M. Zeiher, K. Meinzer, and H. Just, "Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine," *The Lancet*, vol. 338, no. 8782-8783, pp. 1546–1550, 1991.

[76] J.-P. Gratton, P. Bernatchez, and W. C. Sessa, "Caveolae and caveolins in the cardiovascular system," *Circulation Research*, vol. 94, no. 11, pp. 1408–1417, 2004.

[77] C. J. Knowles, M. Dionne, M. Cebova, and I. M. Pinz, "Palmitate-induced translocation of caveolin-3 and endothelial nitric oxide synthase in cardiomyocytes," *Online Journal of Biological Sciences*, vol. 11, no. 2, pp. 27–36, 2011.

[78] G. Sowa, "Caveolae, caveolins, cavins, and endothelial cell function: new insights," *Frontiers in Physiology*, vol. 2, article 120, 2012.

[79] G. García-Cardeña, P. Martasek, B. S. S. Masters et al., "Dissecting the interaction between Nitric Oxide Synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo," *Journal of Biological Chemistry*, vol. 272, no. 41, pp. 25437–25440, 1997.

[80] E. Sbaa, F. Frérart, and O. Feron, "The double regulation of endothelial nitric oxide synthase by caveolae and caveolin: a paradox solved through the study of angiogenesis," *Trends in Cardiovascular Medicine*, vol. 15, no. 5, pp. 157–162, 2005.

[81] J. Saliez, C. Bouzin, G. Rath et al., "Role of caveolar compartmentation in endothelium-derived hyperpolarizing factor-mediated relaxation-ca^{2+} signals and gap junction function are regulated by caveolin in endothelial cells," *Circulation*, vol. 117, no. 8, pp. 1065–1074, 2008.

[82] P. N. Bernatcher, P. M. Bauer, J. Yu, J. S. Prendergast, P. He, and W. C. Sessa, "Dissecting the molecular control of endothelial NO synthase by caveolin-1 using cell-permeable peptides," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 3, pp. 761–766, 2005.

[83] T. Murata, M. I. Lin, R. V. Stan, P. M. Bauer, J. Yu, and W. C. Sessa, "Genetic evidence supporting caveolae microdomain regulation of calcium entry in endothelial cells," *Journal of Biological Chemistry*, vol. 282, no. 22, pp. 16631–16643, 2007.

[84] T. Lockwich, B. B. Singh, X. Liu, and I. S. Ambudkar, "Stabilization of cortical actin induces internalization of Transient Receptor Potential 3 (Trp3)-associated caveolar Ca^{2+} signaling complex and loss of Ca^{2+} influx without disruption of Trp3-inositol trisphosphate receptor association," *Journal of Biological Chemistry*, vol. 276, no. 45, pp. 42401–42408, 2001.

[85] M. T. Nelson, Y. H. Yu Huang, J. E. Brayden, J. Hescheler, and N. B. Standen, "Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels," *Nature*, vol. 344, no. 6268, pp. 770–773, 1990.

[86] M. Félétou and P. M. Vanhoutte, "Endothelium-derived hyperpolarizing factor: where are we now?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 6, pp. 1215–1225, 2006.

[87] J.-F. Jasmin, P. G. Frank, and M. P. Lisanti, *Caveolins and Caveolae—Roles in Signaling and Disease Mechanisms*, Springer, 2012.

[88] H. Chen, J. Yang, P. S. Low, and J.-X. Cheng, "Cholesterol level regulates endosome motility via Rab proteins," *Biophysical Journal*, vol. 94, no. 4, pp. 1508–1520, 2008.

[89] O. Feron and J.-L. Balligand, "Caveolins and the regulation of endothelial nitric oxide synthase in the heart," *Cardiovascular Research*, vol. 69, no. 4, pp. 788–797, 2006.

[90] G. Sowa, "Novel insights into the role of caveolin-2 in cell- and tissue-specific signaling and function," *Biochemistry Research International*, vol. 2011, Article ID 809259, 9 pages, 2011.

[91] D. J. Beech, "Characteristics of transient receptor potential canonical calcium-permeable channels and their relevance to vascular physiology and disease," *Circulation Journal*, vol. 77, no. 3, pp. 570–579, 2013.

[92] A. Dietrich, H. Kalwa, and T. Gudermann, "TRPC channels in vascular cell function," *Thrombosis and Haemostasis*, vol. 103, no. 2, pp. 262–270, 2010.

[93] M. Freichel, S. H. Suh, A. Pfeifer et al., "Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in $TRP4^{-/-}$ mice," *Nature Cell Biology*, vol. 3, no. 2, pp. 121–127, 2001.

[94] T. Yoshida, R. Inoue, T. Morii et al., "Nitric oxide activates TRP channels by cysteine S-nitrosylation," *Nature Chemical Biology*, vol. 2, no. 11, pp. 596–607, 2006.

[95] P. Chaudhuri, S. M. Colles, D. S. Damron, and L. M. Graham, "Lysophosphatidylcholine inhibits endothelial cell migration by increasing intracellular calcium and activating calpain," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 2, pp. 218–223, 2003.

[96] H.-Y. Kwan, Y. Huang, and X. Yao, "Store-operated calcium entry in vascular endothelial cells is inhibited by cGMP via a protein kinase G-dependent mechanism," *Journal of Biological Chemistry*, vol. 275, no. 10, pp. 6758–6163, 2000.

[97] H.-Y. Kwan, Y. Huang, and X. Yao, "Regulation of canonical transient receptor potential isoform 3 (TRPC3) channel by protein kinase G," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 8, pp. 2625–2630, 2004.

[98] M. Poteser, A. Graziani, C. Rosker et al., "TRPC3 and TRPC4 associate to form a redox-sensitive cation channel: evidence for expression of native TRPC3-TRPC4 heteromeric channels in endothelial cells," *Journal of Biological Chemistry*, vol. 281, no. 19, pp. 13588–13595, 2006.

[99] B. A. Miller and W. Zhang, "TRP channels as mediators of oxidative stress," *Advances in Experimental Medicine and Biology*, vol. 704, pp. 531–544, 2011.

[100] M. Chen, T. Masaki, and T. Sawamura, "LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis," *Pharmacology and Therapeutics*, vol. 95, no. 1, pp. 89–100, 2002.

[101] P. E. Szmitko, C.-H. Wang, R. D. Weisel, G. A. Jeffries, T. J. Anderson, and S. Verma, "Biomarkers of vascular disease linking inflammation to endothelial activation part II," *Circulation*, vol. 108, no. 17, pp. 2041–2048, 2003.

[102] I. Andreou, D. Tousoulis, C. Tentolouris, C. Antoniades, and C. Stefanadis, "Potential role of endothelial progenitor cells in the pathophysiology of heart failure: clinical implications and perspectives," *Atherosclerosis*, vol. 189, no. 2, pp. 247–254, 2006.

[103] C. P. Lin, F. Y. Lin, P. H. Huang et al., "Endothelial progenitor cell dysfunction in cardiovascular diseases: role of reactive oxygen species and inflammation," *BioMed Research International*, vol. 2013, Article ID 845037, 10 pages, 2013.

[104] A. Aicher, A. M. Zeiher, and S. Dimmeler, "Mobilizing endothelial progenitor cells," *Hypertension*, vol. 45, no. 3, pp. 321–325, 2005.

[105] H. Iwaguro, J.-I. Yamaguchi, C. Kalka et al., "Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration," *Circulation*, vol. 105, no. 6, pp. 732–738, 2002.

[106] M. Peichev, A. J. Naiyer, D. Pereira et al., "Expression of VEGFR-2 and AC133 by circulating human $CD34^+$ cells identifies a population of functional endothelial precursors," *Blood*, vol. 95, no. 3, pp. 952–958, 2000.

[107] C. Urbich, A. I. de Souza, L. Rossig et al., "Proteomic characterization of human early pro-angiogenic cells," *Journal of Molecular and Cellular Cardiology*, vol. 50, no. 2, pp. 333–336, 2011.

[108] M. E. J. Reinders, T. J. Rabelink, and D. M. Briscoe, "Angiogenesis and endothelial cell repair in renal disease and allograft rejection," *Journal of the American Society of Nephrology*, vol. 17, no. 4, pp. 932–942, 2006.

[109] J. P. Kirton and Q. Xu, "Endothelial precursors in vascular repair," *Microvascular Research*, vol. 79, no. 3, pp. 193–199, 2010.

[110] M. Vasa, S. Fichtlscherer, A. Aicher et al., "Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease," *Circulation Research*, vol. 89, no. 1, pp. E1–E7, 2001.

[111] T. Asahara, T. Murohara, A. Sullivan et al., "Isolation of putative progenitor endothelial cells for angiogenesis," *Science*, vol. 275, no. 5302, pp. 964–967, 1997.

[112] S. Rafii and D. Lyden, "Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration," *Nature Medicine*, vol. 9, no. 6, pp. 702–712, 2003.

[113] E. Gunsilius, H. C. Duba, A. L. Petzer, C. M. Kähler, and G. A. Gastl, "Contribution of endothelial cells of hematopoietic origin to blood vessel formation," *Circulation Research*, vol. 88, no. 1, article E1, 2001.

[114] P. Sarto, E. Balducci, G. Balconi et al., "Effects of exercise training on endothelial progenitor cells in patients with chronic heart failure," *Journal of Cardiac Failure*, vol. 13, no. 9, pp. 701–708, 2007.

[115] C. de Ciuceis, C. Rossini, E. Porteri et al., "Circulating endothelial progenitor cells, microvascular density and fibrosis in obesity before and after bariatric surgery," *Blood Press*, vol. 22, no. 3, pp. 165–172, 2013.

[116] G. M. Rubanyi, "The role of endothelium in cardiovascular homeostasis and diseases," *Journal of Cardiovascular Pharmacology*, vol. 22, supplement 4, pp. S1–S14, 1993.

[117] P. M. Vanhoutte, "Regeneration of the endothelium in vascular injury," *Cardiovascular Drugs and Therapy*, vol. 24, no. 4, pp. 299–303, 2010.

[118] M. S.-K. Wong and P. M. Vanhoutte, "COX-mediated endothelium-dependent contractions: from the past to recent discoveries," *Acta Pharmacologica Sinica*, vol. 31, no. 9, pp. 1095–1102, 2010.

[119] K. Schrör, E. Bretschneider, K. Fischer et al., "Thrombin receptors in vascular smooth muscle cells—function and regulation by vasodilatory prostaglandins," *Thrombosis and Haemostasis*, vol. 103, no. 5, pp. 884–890, 2010.

[120] D. Kerage, D. N. Brindley, and D. G. Hemmings, "Review: novel insights into the regulation of vascular tone by sphingosine 1-phosphate," *Placenta*, vol. 35, pp. S86–S92, 2014.

[121] D. Feng, J. A. Nagy, K. Pyne, H. F. Dvorak, and A. M. Dvorak, "Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP," *Journal of Experimental Medicine*, vol. 187, no. 6, pp. 903–915, 1998.

[122] C. V. Carman and T. A. Springer, "A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them," *The Journal of Cell Biology*, vol. 167, no. 2, pp. 377–388, 2004.

[123] E. C. Butcher, "Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity," *Cell*, vol. 67, no. 6, pp. 1033–1036, 1991.

[124] M. Aurrand-Lions, C. Johnson-Leger, and B. A. Imhof, "The last molecular fortress in leukocyte trans-endothelial migration," *Nature Immunology*, vol. 3, no. 2, pp. 116–118, 2002.

[125] A. R. Pries and W. M. Kuebler, "Normal endothelium," *Handbook of Experimental Pharmacology*, part 1, no. 176, pp. 1–40, 2006.

[126] P. L. A. Giesen, U. Rauch, B. Bohrmann et al., "Blood-borne tissue factor: another view of thrombosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 5, pp. 2311–2315, 1999.

[127] W. C. Aird, "Vascular bed-specific hemostasis: role of endothelium in sepsis pathogenesis," *Critical Care Medicine*, vol. 29, supplement 7, pp. S28–S35, 2001.

[128] Z. Laszik, A. Mitro, F. B. Taylor Jr., G. Ferrell, and C. T. Esmon, "Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway," *Circulation*, vol. 96, no. 10, pp. 3633–3640, 1997.

[129] H. Ishii, H. H. Salem, and C. E. Bell, "Thrombomodulin, an endothelial anticoagulant protein, is absent from the human brain," *Blood*, vol. 67, no. 2, pp. 362–365, 1986.

[130] W. C. Aird, "Endothelial cell heterogeneity," *Critical Care Medicine*, vol. 31, supplement 4, pp. S221–S230, 2003.

[131] S. Verma and T. J. Anderson, "Fundamentals of endothelial function for the clinical cardiologist," *Circulation*, vol. 105, no. 5, pp. 546–549, 2002.

[132] R. Ross and J. A. Glomset, "Atherosclerosis and the arterial smooth muscle cell: proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis," *Science*, vol. 180, no. 4093, pp. 1332–1339, 1973.

[133] W. C. Aird, "Spatial and temporal dynamics of the endothelium," *Journal of Thrombosis and Haemostasis*, vol. 3, no. 7, pp. 1392–1406, 2005.

[134] J. S. Pober and R. S. Cotran, "Cytokines and endothelial cell biology," *Physiological Reviews*, vol. 70, no. 2, pp. 427–451, 1990.

[135] W. Weninger, L. H. Ulfman, G. Cheng et al., "Specialized contributions by α(1,3)-fucosyltransferase-IV and FucT-VII during leukocyte rolling in dermal microvessels," *Immunity*, vol. 12, no. 6, pp. 665–676, 2000.

[136] J. A. Suwaidi, S. Hamasaki, S. T. Higano, R. A. Nishimura, D. R. Holmes Jr., and A. Lerman, "Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction," *Circulation*, vol. 101, no. 9, pp. 948–954, 2000.

[137] V. Schächinger, M. B. Britten, and A. M. Zeiher, "Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease," *Circulation*, vol. 101, no. 16, pp. 1899–1906, 2000.

[138] H. Shimokawa, "Primary endothelial dysfunction: atherosclerosis," *Journal of Molecular and Cellular Cardiology*, vol. 31, no. 1, pp. 23–37, 1999.

[139] I. Ertenli, S. Kiraz, M. A. Öztürk et al., "Plasma fibronectin- and thrombospondin-adhesive molecules during acute attacks and attack-free periods of familial Mediterranean fever," *Rheumatology International*, vol. 20, no. 6, pp. 217–220, 2001.

[140] J. L. Mehta and D. Li, "Identification, regulation and function of a novel lectin-like oxidized low-density lipoprotein receptor," *Journal of the American College of Cardiology*, vol. 39, no. 9, pp. 1429–1435, 2002.

[141] H. Yoshida, N. Kondratenko, S. Green, D. Steinberg, and O. Quehenberger, "Identification of the lectin-like receptor for oxidized low-density lipoprotein in human macrophages and its potential role as a scavenger receptor," *Biochemical Journal*, vol. 334, part 1, pp. 9–13, 1998.

[142] T. Aoyama, M. Chen, H. Fujiwara, T. Masaki, and T. Sawamura, "LOX-1 mediates lysophosphatidylcholine-induced oxidized LDL uptake in smooth muscle cells," *FEBS Letters*, vol. 467, no. 2-3, pp. 217–220, 2000.

[143] S. Mitra, T. Goyal, and J. L. Mehta, "Oxidized LDL, LOX-1 and atherosclerosis," *Cardiovascular Drugs and Therapy*, vol. 25, no. 5, pp. 419–429, 2011.

[144] M. Honjo, K. Nakamura, K. Yamashiro et al., "Lectin-like oxidized LDL receptor-1 is a cell-adhesion molecule involved in endotoxin-induced inflammation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 3, pp. 1274–1279, 2003.

[145] L. Cominacini, A. F. Pasini, U. Garbin et al., "The platelet-endothelium interaction mediated by lectin-like oxidized low-density lipoprotein receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells," *Journal of the American College of Cardiology*, vol. 41, no. 3, pp. 499–507, 2003.

[146] M. Chen, M. Kakutani, M. Minami et al., "Increased expression of lectinlike oxidized low density lipoprotein receptor-1 in initial atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 4, pp. 1107–1115, 2000.

[147] D. Y. Li, H. J. Chen, E. D. Staples et al., "Oxidized low-density lipoprotein receptor LOX-1 and apoptosis in human atherosclerotic lesions," *Journal of Cardiovascular Pharmacology and Therapeutics*, vol. 7, no. 3, pp. 147–153, 2002.

[148] M. Yan, J. L. Mehta, W. Zhang, and C. Hu, "LOX-1, oxidative stress and inflammation: a novel mechanism for diabetic cardiovascular complications," *Cardiovascular Drugs and Therapy*, vol. 25, no. 5, pp. 451–459, 2011.

[149] H. Chen, D. Li, T. Saldeen, and J. L. Mehta, "Transforming growth factor-β1 modulates oxidatively modified LDL-induced expression of adhesion molecules: role of LOX-1," *Circulation Research*, vol. 89, no. 12, pp. 1155–1160, 2001.

[150] X. Wang, M. I. Phillips, and J. L. Mehta, "LOX-1 and angiotensin receptors, and their interplay," *Cardiovascular Drugs and Therapy*, vol. 25, no. 5, pp. 401–417, 2011.

[151] D. Li and J. L. Mehta, "Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells," *Circulation*, vol. 101, no. 25, pp. 2889–2895, 2000.

[152] D. Li and J. L. Mehta, "Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of coronary artery endothelial cells: evidence

from use of antisense LOX-1 mRNA and chemical inhibitors," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 4, pp. 1116–1122, 2000.

[153] M. Kakutani, T. Masaki, and T. Sawamura, "A platelet-endothelium interaction mediated by lectin-like oxidized low-density lipoprotein receptor-1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 1, pp. 360–364, 2000.

[154] A. Rahman, A. L. True, K. N. Anwar, R. D. Ye, T. A. Voyno-Yasenetskaya, and A. B. Malik, "Gαq and G$\beta\gamma$ regulate PAR-1 signaling of thrombin-induced NF-κB activation and ICAM-1 transcription in endothelial cells," *Circulation Research*, vol. 91, no. 5, pp. 398–405, 2002.

[155] S. W. Klarenbach, A. Chipiuk, R. C. Nelson, M. D. Hollenberg, and A. G. Murray, "Differential actions of PAR2 and PAR1, in stimulating human endothelial cell exocytosis and permeability: the role of Rho-GTPases," *Circulation Research*, vol. 92, no. 3, pp. 272–278, 2003.

[156] L. Chi, Y. Li, L. Stehno-Bittel et al., "Interleukin-6 production by endothelial cells via stimulation of protease-activated receptors is amplified by endotoxin and tumor necrosis factor-α," *Journal of Interferon and Cytokine Research*, vol. 21, no. 4, pp. 231–240, 2001.

[157] A. M. Zeiher, H. Drexler, H. Wollschlager, and H. Just, "Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis," *Circulation*, vol. 83, no. 2, pp. 391–401, 1991.

[158] A. M. Zeiher, H. Drexler, B. Saurbier, and H. Just, "Endothelium-mediated coronary blood flow modulation in humans: effects of age, atherosclerosis, hypercholesterolemia, and hypertension," *Journal of Clinical Investigation*, vol. 92, no. 2, pp. 652–662, 1993.

[159] M. A. Creager, J. P. Cooke, M. E. Mendelsohn et al., "Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans," *Journal of Clinical Investigation*, vol. 86, no. 1, pp. 228–234, 1990.

[160] R. J. Esper, R. Machado, J. Vilarino et al., "Endothelium-dependent responses in patients with hypercholesterolemic coronary artery disease under the effects of simvastatin and enalapril, either separately or combined," *American Heart Journal*, vol. 140, no. 4, pp. 684–689, 2000.

[161] R. A. Vogel, M. C. Corretti, and J. Gellman, "Cholesterol, cholesterol lowering, and endothelial function," *Progress in Cardiovascular Diseases*, vol. 41, no. 2, pp. 117–136, 1998.

[162] W.-H. Leung, C.-P. Lau, and C.-K. Wong, "Beneficial effect of cholesterol-lowering therapy on coronary endothelium-dependent relaxation in hypercholesterolaemic patients," *The Lancet*, vol. 341, no. 8859, pp. 1496–1500, 1993.

[163] B. Hornig, V. Maier, and H. Drexler, "Physical training improves endothelial function in patients with chronic heart failure," *Circulation*, vol. 93, no. 2, pp. 210–214, 1996.

[164] D. S. Celermajer, K. E. Sorensen, D. Georgakopoulos et al., "Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults," *Circulation*, vol. 88, no. 5, part 1, pp. 2149–2155, 1993.

[165] D. Urban, S. Ewen, C. Ukena, D. Linz, M. Böhm, and F. Mahfoud, "Treating resistant hypertension: role of renal denervation," *Integrated Blood Pressure Control*, vol. 6, pp. 119–128, 2013.

[166] J. Ketonen, J. Shi, E. Martonen, and E. Mervaala, "Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese C57BL/6 mice," *Circulation Journal*, vol. 74, no. 7, pp. 1479–1487, 2010.

[167] C. Lu, A. X. Zhao, Y.-J. Gao, and R. M. Lee, "Modulation of vein function by perivascular adipose tissue," *European Journal of Pharmacology*, vol. 657, no. 1–3, pp. 111–116, 2011.

[168] A. S. Greenstein, K. Khavandi, S. B. Withers et al., "Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients," *Circulation*, vol. 119, no. 12, pp. 1661–1670, 2009.

[169] T. Szasz, G. F. Bomfim, and R. C. Webb, "The influence of perivascular adipose tissue on vascular homeostasis," *Journal of Vascular Health and Risk Management*, vol. 9, pp. 105–116, 2013.

[170] F. M. Lynch, S. B. Withers, Z. Yao et al., "Perivascular adipose tissue-derived adiponectin activates BK(Ca) channels to induce anticontractile responses," *American Journal of Physiology*, vol. 304, no. 6, pp. H786–H795, 2013.

[171] Y.-J. Gao, Z.-H. Zeng, K. Teoh et al., "Perivascular adipose tissue modulates vascular function in the human internal thoracic artery," *Journal of Thoracic and Cardiovascular Surgery*, vol. 130, no. 4, pp. 1130–1136, 2005.

[172] R. M. Lee, C. Lu, L.-Y. Su, and Y.-J. Gao, "Endothelium-dependent relaxation factor released by perivascular adipose tissue," *Journal of Hypertension*, vol. 27, no. 4, pp. 782–790, 2009.

[173] B. Gálvez-Prieto, B. Somoza, M. Gil-Ortega et al., "Anticontractile effect of perivascular adipose tissue and leptin are reduced in hypertension," *Frontiers in Pharmacology*, vol. 3, article 103, 2012.

[174] Y. Higashi, S. Sukhanov, A. Anwar, S. Y. Shai, and P. Delafontaine, "Aging, atherosclerosis, and IGF-1," *The Journals of Gerontology A*, vol. 67, no. 6, pp. 626–639, 2012.

[175] M. A. Newaz and N. N. A. Nawal, "Effect of α-tocopherol on lipid peroxidation and total antioxidant status in spontaneously hypertensive rats," *American Journal of Hypertension*, vol. 11, no. 12, pp. 1480–1485, 1998.

[176] R. Rezzani, E. Porteri, C. de Ciuceis et al., "Effects of melatonin and pycnogenol on small artery structure and function in spontaneously hypertensive rats," *Hypertension*, vol. 55, no. 6, pp. 1373–1380, 2010.

[177] L. F. Rodella, G. Favero, E. Foglio et al., "Vascular endothelial cells and dysfunctions: role of melatonin," *Frontiers in Bioscience*, vol. 5, pp. 119–129, 2013.

[178] C. M. Kolka and R. N. Bergman, "The endothelium in diabetes: its role in insulin access and diabetic complications," *Reviews in Endocrine and Metabolic Disorders*, vol. 14, no. 1, pp. 13–19, 2013.

[179] F. Giacco and M. Brownlee, "Oxidative stress and diabetic complications," *Circulation Research*, vol. 107, no. 9, pp. 1058–1070, 2010.

[180] R. T. de Jongh, E. H. Serné, R. G. Ijzerman, G. de Vries, and C. D. A. Stehouwer, "Impaired microvascular function in obesity: implications for obesity-associated microangiopathy, hypertension, and insulin resistance," *Circulation*, vol. 109, no. 21, pp. 2529–2535, 2004.

[181] A. E. Caballero, S. Arora, R. Saouaf et al., "Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes," *Diabetes*, vol. 48, no. 9, pp. 1856–1862, 1999.

[182] E. C. Eringa, C. D. A. Stehouwer, M. H. Roos, N. Westerhof, and P. Sipkema, "Selective resistance to vasoactive effects of insulin in muscle resistance arteries of obese Zucker (fa/fa) rats," *American Journal of Physiology*, vol. 293, no. 5, pp. E1134–E1139, 2007.

[183] E. B. Okon, A. W. Y. Chung, P. Rauniyar et al., "Compromised arterial function in human type 2 diabetic patients," *Diabetes*, vol. 54, no. 8, pp. 2415–2423, 2005.

[184] R. I. Meijer, M. P. de Boer, M. R. Groen et al., "Insulin-induced microvascular recruitment in skin and muscle are related and both are associated with whole-body glucose uptake," *Microcirculation*, vol. 19, no. 6, pp. 494–500, 2012.

[185] E. Kostromina, N. Gustavsson, X. Wang et al., "Glucose intolerance and impaired insulin secretion in pancreas-specific signal transducer and activator of transcription-3 knockout mice are associated with microvascular alterations in the pancreas," *Endocrinology*, vol. 151, no. 5, pp. 2050–2059, 2010.

[186] J. S. Yudkin, C. D. A. Stehouwer, J. J. Emeis, and S. W. Coppack, "C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 19, no. 4, pp. 972–978, 1999.

[187] R. Prakash, P. R. Somanath, A. B. El-Remessy et al., "Enhanced cerebral but not peripheral angiogenesis in the Goto-Kakizaki model of type 2 diabetes involves VEGF and peroxynitrite signaling," *Diabetes*, vol. 61, no. 6, pp. 1533–1542, 2012.

[188] S. C. Chang and W. C. V. Yang, "Hyperglycemia induces altered expressions of angiogenesis associated molecules in the trophoblast," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 457971, 11 pages, 2013.

[189] K. H. Elçioğlu, L. Kabasakal, Ş. Çetinel, G. Conturk, S. F. Sezen, and G. Ayanoğlu-Dülger, "Changes in caveolin-1 expression and vasoreactivity in the aorta and corpus cavernosum of fructose and streptozotocin-induced diabetic rats," *European Journal of Pharmacology*, vol. 642, no. 1–3, pp. 113–120, 2010.

[190] E. N. Zeydanli, H. B. Kandilci, and B. Turan, "Doxycycline ameliorates vascular endothelial and contractile dysfunction in the thoracic aorta of diabetic rats," *Cardiovascular Toxicology*, vol. 11, no. 2, pp. 134–147, 2011.

[191] S. M. Shenouda, M. E. Widlansky, K. Chen et al., "Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus," *Circulation*, vol. 124, no. 4, pp. 444–453, 2011.

[192] A. B. Goldfine, J. A. Beckman, R. A. Betensky et al., "Family history of diabetes is a major determinant of endothelial function," *Journal of the American College of Cardiology*, vol. 47, no. 12, pp. 2456–2461, 2006.

[193] R. I. Meijer, E. H. Serne, Y. M. Smulders, V. W. M. van Hinsbergh, J. S. Yudkin, and E. C. Eringa, "Perivascular adipose tissue and its role in type 2 diabetes and cardiovascular disease," *Current Diabetes Reports*, vol. 11, no. 3, pp. 211–217, 2011.

[194] S. Hagita, M. Osaka, K. Shimokado, and M. Yoshida, "Adipose inflammation initiates recruitment of leukocytes to mouse femoral artery: role of adipo-vascular axis in chronic inflammation," *PLoS ONE*, vol. 6, no. 5, Article ID e19871, 2011.

[195] F. Kim, M. Pham, E. Maloney et al., "Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 11, pp. 1982–1988, 2008.

[196] E. C. Eringa, C. D. A. Stehouwer, K. Walburg et al., "Physiological concentrations of insulin induce endothelin-dependent vasoconstriction of skeletal muscle resistance arteries in the presence of tumor necrosis factor-α dependence on c-Jun N-terminal kinase," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 2, pp. 274–280, 2006.

[197] W. Bakker, P. Sipkema, C. D. A. Stehouwer et al., "Protein kinase C θ activation induces insulin-mediated constriction of muscle resistance arteries," *Diabetes*, vol. 57, no. 3, pp. 706–713, 2008.

[198] R. I. Meijer, W. Bakker, C. L. Alta et al., "Perivascular adipose tissue control of insulin-induced vasoreactivity in muscle is impaired in db/db mice," *Diabetes*, vol. 62, no. 2, pp. 590–598, 2013.

[199] C. Rask-Madsen, H. Domínguez, N. Ihlemann, T. Hermann, L. Køber, and C. Torp-Pedersen, "Tumor necrosis factor-α inhibits insulin's stimulating effect on glucose uptake and endothelium-dependent vasodilation in humans," *Circulation*, vol. 108, no. 15, pp. 1815–1821, 2003.

[200] A. J. Houben, E. C. Eringa, A. M. Jonk, E. H. Serne, Y. M. Smulders, and C. D. Stehouwer, "Perivascular fat and the microcirculation: relevance to insulin resistance, diabetes, and cardiovascular disease," *Current Cardiovascular Risk Reports* , vol. 6, no. 1, pp. 80–90, 2012.

[201] T. K. Chatterjee, L. L. Stoll, G. M. Denning et al., "Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding," *Circulation Research*, vol. 104, no. 4, pp. 541–549, 2009.

[202] E. Henrichot, C. E. Juge-Aubry, A. Pernin et al., "Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 12, pp. 2594–2599, 2005.

[203] S. B. Withers, C. Agabiti-Rosei, D. M. Livingstone et al., "Macrophage activation is responsible for loss of anticontractile function in inflamed perivascular fat," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 4, pp. 908–913, 2011.

[204] N. Hosogai, A. Fukuhara, K. Oshima et al., "Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation," *Diabetes*, vol. 56, no. 4, pp. 901–911, 2007.

[205] J. Yin, Z. Gao, Q. He, D. Zhou, Z. Guo, and J. Ye, "Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue," *American Journal of Physiology*, vol. 296, no. 2, pp. E333–E342, 2009.

[206] N. K. Ghaisas, J. B. Foley, D. S. O'Briain, P. Crean, D. Kelleher, and M. Walsh, "Adhesion molecules in nonrheumatic aortic valve disease: endothelial expression, serum levels and effects of valve replacement," *Journal of the American College of Cardiology*, vol. 36, no. 7, pp. 2257–2262, 2000.

[207] A. M. Müller, C. Cronen, L. I. Kupferwasser, H. Oelert, K. M. Müller, and C. J. Kirkpatrick, "Expression of endothelial cell adhesion molecules on heart valves: up-regulation in degeneration as well as acute endocarditis," *Journal of Pathology*, vol. 191, no. 1, pp. 54–60, 2000.

[208] F. Chalajour, H. Treede, A. Ebrahimnejad, H. Lauke, H. Reichenspurner, and S. Ergun, "Angiogenic activation of valvular endothelial cells in aortic valve stenosis," *Experimental Cell Research*, vol. 298, no. 2, pp. 455–464, 2004.

[209] J. P. J. Halcox and A. A. Quyyumi, "Coronary vascular endothelial function and myocardial ischemia: why should we worry about endothelial dysfunction?" *Coronary Artery Disease*, vol. 12, no. 6, pp. 475–484, 2001.

[210] J. F. Argacha, D. Fontaine, D. Adamopoulos et al., "Acute effect of sidestream cigarette smoke extract on vascular endothelial function," *Journal of Cardiovascular Pharmacology*, vol. 52, no. 3, pp. 262–267, 2008.

[211] P. Balakumar, R. Sharma, and M. Singh, "Benfotiamine attenuates nicotine and uric acid-induced vascular endothelial dysfunction in the rat," *Pharmacological Research*, vol. 58, no. 5-6, pp. 356–363, 2008.

[212] S. C. Campbell, R. J. Moffatt, and B. A. Stamford, "Smoking and smoking cessation—the relationship between cardiovascular disease and lipoprotein metabolism: a review," *Atherosclerosis*, vol. 201, no. 2, pp. 225–235, 2008.

[213] P. Balakumar and J. Kaur, "Is nicotine a key player or spectator in the induction and progression of cardiovascular disorders?" *Pharmacological Research*, vol. 60, no. 5, pp. 361–368, 2009.

[214] R. M. Pittilo, "Cigarette smoking and endothelial injury: a review," *Advances in Experimental Medicine and Biology*, vol. 273, pp. 61–78, 1990.

[215] D. Bernhard, A. Rossmann, and G. Wick, "Metals in cigarette smoke," *IUBMB Life*, vol. 57, no. 12, pp. 805–809, 2005.

[216] M. M. Rahman and I. Laher, "Structural and functional alteration of blood vessels caused by cigarette smoking: an overiew of molecular mechanisms," *Current Vascular Pharmacology*, vol. 5, no. 4, pp. 276–292, 2007.

[217] H.-L. Luo, W.-J. Zang, J. Lu, X.-J. Yu, Y.-X. Lin, and Y.-X. Cao, "The protective effect of captopril on nicotine-induced endothelial dysfunction in rat," *Basic and Clinical Pharmacology and Toxicology*, vol. 99, no. 3, pp. 237–245, 2006.

[218] C. R. W. Kuhlmann, J. R. F. C. Trümper, H. Tillmanns, C. A. Schaefer, and A. Erdogan, "Nicotine inhibits large conductance Ca^{2+}-activated K^+ channels and the NO/-cGMP signaling pathway in cultured human endothelial cells," *Scandinavian Cardiovascular Journal*, vol. 39, no. 6, pp. 348–352, 2005.

[219] L. Erhardt, "Cigarette smoking: an undertreated risk factor for cardiovascular disease," *Atherosclerosis*, vol. 205, no. 1, pp. 23–32, 2009.

[220] L. F. Rodella, C. Rossini, G. Favero, E. Foglio, C. Loreto, and R. Rezzani, "Nicotine-induced morphological changes in rat aorta: the protective role of melatonin," *Cells Tissues Organs*, vol. 195, no. 3, pp. 252–259, 2012.

[221] L. F. Rodella, G. Favero, C. Rossini, E. Foglio, R. J. Reiter, and R. Rezzani, "Endothelin-1 as a potential marker of melatonin's therapeutic effects in smoking-induced vasculopathy," *Life Sciences*, vol. 87, no. 17-18, pp. 558–564, 2010.

[222] X. L. Wang, M. Raveendran, and J. Wang, "Genetic influence on cigarette-induced cardiovascular disease," *Progress in Cardiovascular Diseases*, vol. 45, no. 5, pp. 361–382, 2003.

[223] M. D. Herrera, C. Mingorance, R. Rodríguez-Rodríguez, and M. A. de Sotomayor, "Endothelial dysfunction and aging: an update," *Ageing Research Reviews*, vol. 9, no. 2, pp. 142–152, 2010.

[224] A. U. Ferrari, A. Radaelli, and M. Centola, "Invited review: aging and the cardiovascular system," *Journal of Applied Physiology*, vol. 95, no. 6, pp. 2591–2597, 2003.

[225] R. L. Matz and R. Andriantsitohaina, "Age-related endothelial dysfunction: potential implications for pharmacotherapy," *Drugs and Aging*, vol. 20, no. 7, pp. 527–550, 2003.

[226] R. P. Brandes, I. Fleming, and R. Busse, "Endothelial aging," *Cardiovascular Research*, vol. 66, no. 2, pp. 286–294, 2005.

[227] A. A. Puca, A. Carrizzo, F. Villa et al., "Vascular ageing: the role of oxidative stress," *The International Journal of Biochemistry & Cell Biology*, vol. 45, no. 3, pp. 556–559, 2013.

[228] S. M. L. Wallace, Y. Yasmin, C. M. McEniery et al., "Isolated systolic hypertension is characterized by increased aortic stiffness and endothelial dysfunction," *Hypertension*, vol. 50, no. 1, pp. 228–233, 2007.

[229] A. Csiszar, J. Toth, J. Peti-Peterdi, and Z. Ungvari, "The aging kidney: role of endothelial oxidative stress and inflammation," *Acta Physiologica Hungarica*, vol. 94, no. 1-2, pp. 107–115, 2007.

[230] J. M. Price, A. Hellermann, G. Hellermann, and E. T. Sutton, "Aging enhances vascular dysfunction induced by the Alzheimer's peptide β-amyloid," *Neurological Research*, vol. 26, no. 3, pp. 305–311, 2004.

[231] T. Kunieda, T. Minamino, K. Miura et al., "Reduced nitric oxide causes age-associated impairment of circadian rhythmicity," *Circulation Research*, vol. 102, no. 5, pp. 607–614, 2008.

[232] A. L. Burnett, "The role of nitric oxide in erectile dysfunction: implications for medical therapy," *Journal of Clinical Hypertension*, vol. 8, supplement 4, no. 12, pp. 53–62, 2006.

[233] M. Sanada, A. Taguchi, Y. Higashi et al., "Forearm endothelial function and bone mineral loss in postmenopausal women," *Atherosclerosis*, vol. 176, no. 2, pp. 387–392, 2004.

[234] H. R. Coleman, C.-C. Chan, F. L. Ferris III, and E. Y. Chew, "Age-related macular degeneration," *The Lancet*, vol. 372, no. 9652, pp. 1835–1845, 2008.

[235] P. E. Gates, W. D. Strain, and A. C. Shore, "Human endothelial function and microvascular ageing," *Experimental Physiology*, vol. 94, no. 3, pp. 311–316, 2009.

[236] J. Lähteenvuo and A. Rosenzweig, "Effects of aging on angiogenesis," *Circulation Research*, vol. 110, no. 9, pp. 1252–1264, 2012.

[237] T. Finkel, "Oxidant signals and oxidative stress," *Current Opinion in Cell Biology*, vol. 15, no. 2, pp. 247–254, 2003.

[238] C. Vecchione and R. P. Brandes, "Withdrawal of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors elicits oxidative stress and induces endothelial dysfunction in mice," *Circulation Research*, vol. 91, no. 2, pp. 173–179, 2002.

[239] Z. Ungvari, L. Bailey-Downs, D. Sosnowska et al., "Vascular oxidative stress in aging: a homeostatic failure due to dysregulation of NRF2-mediated antioxidant response," *American Journal of Physiology*, vol. 301, no. 2, pp. H363–H372, 2011.

[240] D.-F. Dai, P. S. Rabinovitch, and Z. Ungvari, "Mitochondria and cardiovascular aging," *Circulation Research*, vol. 110, no. 8, pp. 1109–1124, 2012.

[241] B. L. Tang, "Sirt1's systemic protective roles and its promise as a target in antiaging medicine," *Translational Research*, vol. 157, no. 5, pp. 276–284, 2011.

[242] S. Chung, H. Yao, S. Caito, J.-W. Hwang, G. Arunachalam, and I. Rahman, "Regulation of SIRT1 in cellular functions: role of polyphenols," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 79–90, 2010.

[243] A. M. Cuevas and A. M. Germain, "Diet and endothelial function," *Biological Research*, vol. 37, no. 2, pp. 225–230, 2004.

[244] S.-I. Yamagishi, T. Matsui, and K. Nakamura, "Atheroprotective properties of Pigment Epithelium-Derived Factor (PEDF) in cardiometabolic disorders," *Current Pharmaceutical Design*, vol. 15, no. 9, pp. 1027–1033, 2009.

[245] D. Ziegler, "Type 2 diabetes as an inflammatory cardiovascular disorder," *Current Molecular Medicine*, vol. 5, no. 3, pp. 309–322, 2005.

[246] J. Szostak and P. Laurant, "The forgotten face of regular physical exercise: a "natural" anti-atherogenic activity," *Clinical Science*, vol. 121, no. 3, pp. 91–106, 2011.

[247] M. D. Brown and D. L. Feairheller, "Are there race-dependent endothelial cell responses to exercise?" *Exercise & Sport Sciences Reviews*, vol. 41, no. 1, pp. 44–54, 2013.

[248] K. E. Flynn, I. L. Piña, D. J. Whellan et al., "Effects of exercise training on health status in patients with chronic heart failure HF-ACTION randomized controlled trial," *Journal of the American Medical Association*, vol. 301, no. 14, pp. 1451–1459, 2009.

[249] J. Downing and G. J. Balady, "The role of exercise training in heart failure," *Journal of the American College of Cardiology*, vol. 58, no. 6, pp. 561–569, 2011.

[250] J. Niebauer, "Effects of exercise training on inflammatory markers in patients with heart failure," *Heart Failure Reviews*, vol. 13, no. 1, pp. 39–49, 2008.

[251] K. C. Miranda, T. Huynh, Y. Tay et al., "A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes," *Cell*, vol. 126, no. 6, pp. 1203–1217, 2006.

[252] M. M. Fernandes-Silva, V. O. Carvalho, G. V. Guimarães, F. Bacal, and E. A. Bocchi, "Physical exercise and microRNAs: new frontiers in heart failure," *Arquivos Brasileiros de Cardiologia*, vol. 98, no. 5, pp. 459–466, 2012.

[253] R. A. Vogel, "The Mediterranean diet and endothelial function: why some dietary fats may be healthy," *Cleveland Clinic Journal of Medicine*, vol. 67, no. 4, article 232, pp. 235–236, 2000, Erratum in *Cleveland Clinic Journal of Medicine*, vol. 67, no. 7, article 467, 2000.

[254] N. Nicolaïew, N. Lemort, L. Adorni et al., "Comparison between extra virgin olive oil and oleic acid rich sunflower oil: effects on postprandial lipemia and LDL susceptibility to oxidation," *Annals of Nutrition and Metabolism*, vol. 42, no. 5, pp. 251–260, 1998.

[255] E. Lopez-Garcia, M. B. Schulze, J. B. Meigs et al., "Consumption of trans fatty acids is related to plasma biomarkers of inflammation and endothelial dysfunction," *Journal of Nutrition*, vol. 135, no. 3, pp. 562–566, 2005.

[256] J. M. Silva and C. Saldanha, "Diet, atherosclerosis and atherothrombotic events," *Revista Portuguesa de Cardiologia*, vol. 26, no. 3, pp. 277–294, 2007.

[257] F. B. Hu, J. E. Manson, and W. C. Willett, "Types of dietary fat and risk of coronary heart disease: a critical review," *Journal of the American College of Nutrition*, vol. 20, no. 1, pp. 5–19, 2001.

[258] D. Mozaffarian, "Trans fatty acids—effects on systemic inflammation and endothelial function," *Atherosclerosis Supplements*, vol. 7, no. 2, pp. 29–32, 2006.

[259] K. Esposito, R. Marfella, M. Ciotola et al., "Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial," *Journal of the American Medical Association*, vol. 292, no. 12, pp. 1440–1446, 2004.

[260] R. Estruch, M. A. Martínez-González, D. Corella et al., "Effects of a Mediterranean-style diet on cardiovascular risk factors a randomized trial," *Annals of Internal Medicine*, vol. 145, no. 1, pp. 1–11, 2006.

[261] M. Ryan, D. Mcinerney, D. Owens, P. Collins, A. Johnson, and G. H. Tomkin, "Diabetes and the Mediterranean diet: a beneficial effect of oleic acid on insulin sensitivity, adipocyte glucose transport and endothelium-dependent vasoreactivity," *Monthly Journal of the Association of Physicians*, vol. 93, no. 2, pp. 85–91, 2000.

[262] M. de Lorgeril, P. Salen, J.-L. Martin, I. Monjaud, J. Delaye, and N. Mamelle, "Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart study," *Circulation*, vol. 99, no. 6, pp. 779–785, 1999.

[263] W. J. E. Bemelmans, J. D. Lefrandt, E. J. M. Feskens et al., "Change in saturated fat intake is associated with progression of carotid and femoral intima-media thickness, and with levels of soluble intercellular adhesion molecule-1," *Atherosclerosis*, vol. 163, no. 1, pp. 113–120, 2002.

[264] A. Michalsen, N. Lehmann, C. Pithan et al., "Mediterranean diet has no effect on markers of inflammation and metabolic risk factors in patients with coronary artery disease," *European Journal of Clinical Nutrition*, vol. 60, no. 4, pp. 478–485, 2006.

[265] P. J. L. Ong, T. S. Dean, C. S. Hayward, P. L. D. Monica, T. A. B. Sanders, and P. Collins, "Effect of fat and carbohydrate consumption on endothelial function," *The Lancet*, vol. 354, no. 9196, article 2134, 1999.

[266] B. Khalfoun, G. Thibault, P. Bardos, and Y. Lebranchu, "Docosahexaenoic and eicosapentaenoic acids inhibit in vitro human lymphocyte-endothelial cell adhesion," *Transplantation*, vol. 62, no. 11, pp. 1649–1657, 1996.

[267] C. T. Kappagoda, D. A. Hyson, and E. A. Amsterdam, "Low-carbohydrate-high-protein diets: is there a place for them in clinical cardiology?" *Journal of the American College of Cardiology*, vol. 43, no. 5, pp. 725–730, 2004.

[268] E. C. Westman, W. S. Yancy Jr., M. K. Olsen, T. Dudley, and J. R. Guyton, "Effect of a low-carbohydrate, ketogenic diet program compared to a low-fat diet on fasting lipoprotein subclasses," *International Journal of Cardiology*, vol. 110, no. 2, pp. 212–216, 2006.

[269] W. E. Connor and S. L. Connor, "Should a low-fat, high-carbohydrate diet be recommended for everyone?" *The New England Journal of Medicine*, vol. 337, no. 8, pp. 562–563, 1997.

[270] M. B. Katan, S. M. Grundy, and W. C. Willett, "Beyond low-fat diets," *The New England Journal of Medicine*, vol. 337, no. 8, pp. 563–566, 1997.

[271] S. L. Weinberg, "The diet-heart hypothesis: a critique," *Journal of the American College of Cardiology*, vol. 43, no. 5, pp. 731–733, 2004.

[272] R. Landberg, N. Naidoo, and R. M. van Dam, "Diet and endothelial function: from individual components to dietary patterns," *Current Opinion in Lipidology*, vol. 23, no. 2, pp. 147–155, 2012.

[273] R. A. Vogel, M. C. Corretti, and G. D. Plotnick, "Effect of a single high-fat meal on endothelial function in healthy subjects," *American Journal of Cardiology*, vol. 79, no. 3, pp. 350–354, 1997.

[274] C. Marin, R. Ramirez, J. Delgado-Lista et al., "Mediterranean diet reduces endothelial damage and improves the regenerative capacity of endothelium," *American Journal of Clinical Nutrition*, vol. 93, no. 2, pp. 267–274, 2011.

[275] U. Scherrer, D. Randin, P. Vollenweider, L. Vollenweider, and P. Nicod, "Nitric oxide release accounts for insulin's vascular effects in humans," *Journal of Clinical Investigation*, vol. 94, no. 6, pp. 2511–2515, 1994.

[276] F. Pérez-Jiménez, P. Castro, J. López-Miranda et al., "Circulating levels of endothelial function are modulated by dietary monounsaturated fat," *Atherosclerosis*, vol. 145, no. 2, pp. 351–358, 1999.

[277] F. Fuentes, J. López-Miranda, E. Sánchez et al., "Mediterranean and low-fat diets improve endothelial function in hypercholesterolemic men," *Annals of Internal Medicine*, vol. 134, no. 12, pp. 1115–1119, 2001.

[278] F. Leighton, S. Miranda-Rottmann, and I. Urquiaga, "A central role of eNOS in the protective effect of wine against metabolic syndrome," *Cell Biochemistry and Function*, vol. 24, no. 4, pp. 291–298, 2006.

[279] D. Grassi, G. Desideri, and C. Ferri, "Flavonoids: antioxidants against atherosclerosis," *Nutrients*, vol. 2, no. 8, pp. 889–902, 2010.

[280] R. Rezzani, L. F. Rodella, S. Tengattini et al., "Protective role of polyphenols in cyclosporine A-induced nephrotoxicity during rat pregnancy," *Journal of Histochemistry and Cytochemistry*, vol. 54, no. 8, pp. 923–932, 2006.

[281] A. T. Dinkova-Kostova and P. Talalay, "Direct and indirect antioxidant properties of inducers of cytoprotective proteins," *Molecular Nutrition and Food Research*, vol. 52, supplement 1, pp. S128–S138, 2008.

[282] A. T. Dinkova-Kostova, J. Cheah, A. Samouilov et al., "Phenolic Michael reaction acceptors: combined direct and indirect antioxidant defenses against electrophiles and oxidants," *Medicinal Chemistry*, vol. 3, no. 3, pp. 261–268, 2007.

[283] M.-L. Hu, "Dietary polyphenols as antioxidants and anticancer agents: more questions than answers," *Chang Gung Medical Journal*, vol. 34, no. 5, pp. 449–460, 2011.

[284] B. Holst and G. Williamson, "Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants," *Current Opinion in Biotechnology*, vol. 19, no. 2, pp. 73–82, 2008.

[285] L. Badimon, G. Vilahur, and T. Padro, "Nutraceuticals and atherosclerosis: human trials," *Cardiovascular Therapeutics*, vol. 28, no. 4, pp. 202–215, 2010.

[286] M. Kyaw, M. Yoshizumi, K. Tsuchiya, Y. Izawa, Y. Kanematsu, and T. Tamaki, "Atheroprotective effects of antioxidants through inhibition of mitogen-activated protein kinases," *Acta Pharmacologica Sinica*, vol. 25, no. 8, pp. 977–985, 2004.

[287] R. Rezzani, S. Tengattini, F. Bonomini et al., "Red wine polyphenols prevent cyclosporine-induced nephrotoxicity at the level of the intrinsic apoptotic pathway," *Physiological Research*, vol. 58, no. 4, pp. 511–519, 2009.

[288] C. Morand, C. Dubray, D. Milenkovic et al., "Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers," *American Journal of Clinical Nutrition*, vol. 93, no. 1, pp. 73–80, 2011.

[289] S. J. Duffy, J. F. Keaney Jr., M. Holbrook et al., "Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease," *Circulation*, vol. 104, no. 2, pp. 151–156, 2001.

[290] M.-I. Covas, "Olive oil and the cardiovascular system," *Pharmacological Research*, vol. 55, no. 3, pp. 175–186, 2007.

[291] J. S. Perona, R. Cabello-Moruno, and V. Ruiz-Gutierrez, "The role of virgin olive oil components in the modulation of endothelial function," *Journal of Nutritional Biochemistry*, vol. 17, no. 7, pp. 429–445, 2006.

[292] D. J. Hausenloy and D. M. Yellon, "Targeting residual cardiovascular risk: raising high-density lipoprotein cholesterol levels," *Postgraduate Medical Journal*, vol. 84, no. 997, pp. 590–598, 2008.

[293] M. A. Carluccio, M. Massaro, E. Scoditti, and R. de Caterina, "Vasculoprotective potential of olive oil components," *Molecular Nutrition and Food Research*, vol. 51, no. 10, pp. 1225–1234, 2007.

[294] A. Marquez-Martin, R. D. L. Puerta, A. Fernandez-Arche, V. Ruiz-Gutierrez, and P. Yaqoob, "Modulation of cytokine secretion by pentacyclic triterpenes from olive pomace oil in human mononuclear cells," *Cytokine*, vol. 36, no. 5-6, pp. 211–217, 2006.

[295] U. Simonsen, R. Rodriguez-Rodriguez, T. Dalsgaard, N. H. Buus, and E. Stankevicius, "Novel approaches to improving endothelium-dependent nitric oxide-mediated vasodilatation," *Pharmacological Reports*, vol. 61, no. 1, pp. 105–115, 2009.

[296] R. Masella, R. Varì, M. D'Archivio et al., "Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes," *Journal of Nutrition*, vol. 134, no. 4, pp. 785–791, 2004.

[297] R. Masella, R. di Benedetto, R. Varì, C. Filesi, and C. Giovannini, "Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes," *Journal of Nutritional Biochemistry*, vol. 16, no. 10, pp. 577–586, 2005.

[298] M. A. Carluccio, M. Massaro, C. Bonfrate et al., "Oleic acid inhibits endothelial activation: a direct vascular antiatherogenic mechanism of a nutritional component in the Mediterranean diet," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 19, no. 2, pp. 220–228, 1999.

[299] M. Massaro, M. A. Carluccio, A. Paolicchi, F. Bosetti, G. Solaini, and R. de Caterina, "Mechanisms for reduction of endothelial activation by oleate: inhibition of nuclear factorκB through antioxidant effects," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 67, no. 2-3, pp. 175–181, 2002.

[300] P. Yaqoob, E. A. Newsholme, and P. C. Calder, "Inhibition of natural killer cell activity by dietary lipids," *Immunology Letters*, vol. 41, no. 2-3, pp. 241–247, 1994.

[301] P. Yaqoob, E. A. Newsholme, and P. C. Calder, "The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation," *Immunology*, vol. 82, no. 4, pp. 603–610, 1994.

[302] S. Tsimikas, A. Philis-Tsimikas, S. Alexopoulos, F. Sigari, C. Lee, and P. D. Reaven, "LDL isolated from Greek subjects on a typical diet or from American subjects on an oleate-supplemented diet induces less monocyte chemotaxis and adhesion when exposed to oxidative stress," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 19, no. 1, pp. 122–130, 1999.

[303] J. Ruano, J. Lopez-Miranda, F. Fuentes et al., "Phenolic content of virgin olive oil improves ischemic reactive hyperemia in hypercholesterolemic patients," *Journal of the American College of Cardiology*, vol. 46, no. 10, pp. 1864–1868, 2005.

[304] M. Fitó, R. de La Torre, and M.-I. Covas, "Olive oil and oxidative stress," *Molecular Nutrition and Food Research*, vol. 51, no. 10, pp. 1215–1224, 2007.

[305] G. Lippi, M. Franchini, E. J. Favaloro, and G. Targher, "Moderate red wine consumption and cardiovascular disease risk: beyond the French paradox," *Seminars in Thrombosis and Hemostasis*, vol. 36, no. 1, pp. 59–70, 2010.

[306] P. Gresele, C. Cerletti, G. Guglielmini, P. Pignatelli, G. de Gaetano, and F. Violi, "Effects of resveratrol and other wine polyphenols on vascular function: an update," *Journal of Nutritional Biochemistry*, vol. 22, no. 3, pp. 201–211, 2011.

[307] P. Bališ, A. Púzserová, P. Slezák, N. Sestáková, O. Pecháňová, and I. Bernátová, "Short-term administration of alibernet red wine extract failed to affect blood pressure and to improve endothelial function in young normotensive and spontaneously hypertensive rats," *Physiological Research*, vol. 62, no. 6, pp. 631–641, 2013.

[308] J. Bornhoeft, D. Castaneda, T. Nemoseck, P. Wang, S. M. Henning, and M. Y. Hong, "The protective effects of green tea polyphenols: lipid profile, inflammation, and antioxidant capacity in rats fed an atherogenic diet and dextran sodium sulfate," *Journal of Medicinal Food*, vol. 15, no. 8, pp. 726–732, 2012.

[309] S. V. Nigdikar, N. R. Williams, B. A. Griffin, and A. N. Howard, "Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation in vivo,"

American Journal of Clinical Nutrition, vol. 68, no. 2, pp. 258–265, 1998.

[310] A. Agouni, A.-H. Lagrue-Lak-Hal, H. A. Mostefai et al., "Red wine polyphenols prevent metabolic and cardiovascular alterations associated with obesity in Zucker fatty rats (Fa/Fa)," *PLoS ONE*, vol. 4, no. 5, Article ID e5557, 2009.

[311] G. Scola, D. Conte, P. W. D.-S. Spada et al., "Flavan-3-ol compounds from wine wastes with in vitro and in vivo antioxidant activity," *Nutrients*, vol. 2, no. 10, pp. 1048–1059, 2010.

[312] M. G. Lionetto, M. E. Giordano, A. Calisi, E. Erroi, F. de Nuccio, and T. Schettino, "Effect of the daily ingestion of a purified anthocyanin extract from grape skin on rat serum antioxidant capacity," *Physiological Research*, vol. 60, no. 4, pp. 637–645, 2011.

[313] T. Wallerath, G. Deckert, T. Ternes et al., "Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase," *Circulation*, vol. 106, no. 13, pp. 1652–1658, 2002.

[314] K. Karatzi, E. Karatzis, C. Papamichael, J. Lekakis, and A. Zampelas, "Effects of red wine on endothelial function: postprandial studies vs clinical trials," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 19, no. 10, pp. 744–750, 2009.

[315] J. G. Zou, Z. R. Wang, Y. Z. Huang, K. J. Cao, and J. M. Wu, "Effect of red wine and wine polyphenol resveratrol on endothelial function in hypercholesterolemic rabbits," *International Journal of Molecular Medicine*, vol. 11, no. 3, pp. 317–320, 2003.

[316] R. Jiménez, R. López-Sepúlveda, M. Kadmiri et al., "Polyphenols restore endothelial function in DOCA-salt hypertension: role of endothelin-1 and NADPH oxidase," *Free Radical Biology and Medicine*, vol. 43, no. 3, pp. 462–473, 2007.

[317] A. Cavallaro, T. Ainis, C. Bottari, and V. Fimiani, "Effect of resveratrol on some activities of isolated and in whole blood human neutrophils," *Physiological Research*, vol. 52, no. 5, pp. 555–562, 2003.

[318] S. K. Nicholson, G. A. Tucker, and J. M. Brameld, "Effects of dietary polyphenols on gene expression in human vascular endothelial cells," *Proceedings of the Nutrition Society*, vol. 67, no. 1, pp. 42–47, 2008.

[319] R. Corder, J. A. Douthwaite, D. M. Lees et al., "Endothelin-1 synthesis red wine," *Nature*, vol. 414, no. 6866, pp. 863–864, 2001.

[320] L. F. Rodella, L. Vanella, S. J. Peterson et al., "Heme oxygenase-derived carbon monoxide restores vascular function in type 1 diabetes," *Drug Metabolism Letters*, vol. 2, no. 4, pp. 290–300, 2008.

[321] S. Hamed, J. Alshiek, A. Aharon, B. Brenner, and A. Roguin, "Red wine consumption improves in vitro migration of endothelial progenitor cells in young, healthy individuals," *American Journal of Clinical Nutrition*, vol. 92, no. 1, pp. 161–169, 2010.

[322] G. Chiva-Blanch, M. Urpi-Sarda, E. Ros et al., "Dealcoholized red wine decreases systolic and diastolic blood pressure and increases plasma nitric oxide: short communication," *Circulation Research*, vol. 111, no. 8, pp. 1065–1068, 2012.

[323] J. H. Stein, J. G. Keevil, D. A. Wiebe, S. Aeschlimann, and J. D. Folts, "Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease," *Circulation*, vol. 100, no. 10, pp. 1050–1055, 1999.

[324] A. C. M. Andrade, F. H. Y. Cesena, F. M. Consolim-Colombo et al., "Short-term red wine consumption promotes differential effects on plasma levels of high-density lipoprotein cholesterol, sympathetic activity, and endothelial function in hypercholesterolemic, hypertensive, and healthy subjects," *Clinics*, vol. 64, no. 5, pp. 435–442, 2009.

[325] P.-H. Huang, Y.-H. Chen, H.-Y. Tsai et al., "Intake of red wine increases the number and functional capacity of circulating endothelial progenitor cells by enhancing nitric oxide bioavailability," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 4, pp. 869–877, 2010.

[326] C. D. Venkov, P. R. Myers, M. A. Tanner, M. Su, and D. E. Vaughan, "Ethanol increases endothelial nitric oxide production through modulation of nitric oxide synthase expression," *Thrombosis and Haemostasis*, vol. 81, no. 4, pp. 638–642, 1999.

[327] J. F. Leikert, T. R. Räthel, P. Wohlfart, V. Cheynier, A. M. Vollmar, and V. M. Dirsch, "Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells," *Circulation*, vol. 106, no. 13, pp. 1614–1617, 2002.

[328] A. M. Cuevas, V. Guasch, O. Castillo et al., "A high-fat diet induces and red wine counteracts endothelial dysfunction in human volunteers," *Lipids*, vol. 35, no. 2, pp. 143–148, 2000.

[329] A. A. Quyyumi, "Prognostic value of endothelial function," *American Journal of Cardiology*, vol. 91, no. 12, pp. 19H–24H, 2003.

[330] T. Neunteufl, S. Heher, R. Katzenschlager et al., "Late prognostic value of flow-mediated dilation in the brachial artery of patients with chest pain," *American Journal of Cardiology*, vol. 86, no. 2, pp. 207–210, 2000.

[331] J. Lefèvre, S.-É. Michaud, P. Haddad et al., "Moderate consumption of red wine (cabernet sauvignon) improves ischemia-induced neovascularization in ApoE-deficient mice: effect on endothelial progenitor cells and nitric oxide," *FASEB Journal*, vol. 21, no. 14, pp. 3845–3852, 2007.

[332] P. A. Cahill and E. M. Redmond, "Alcohol and cardiovascular disease-modulation of vascular cell function," *Nutrients*, vol. 4, no. 4, pp. 297–318, 2012.

[333] A. P. Whelan, W. H. F. Sutherland, M. P. McCormick, D. J. Yeoman, S. A. de Jong, and M. J. A. Williams, "Effects of white and red wine on endothelial function in subjects with coronary artery disease," *Internal Medicine Journal*, vol. 34, no. 5, pp. 224–228, 2004.

[334] S. Agewall, S. Wright, R. N. Doughty, G. A. Whalley, M. Duxbury, and N. Sharpe, "Does a glass of red wine improve endothelial function?" *European Heart Journal*, vol. 21, no. 1, pp. 74–78, 2000.

[335] M. Hashimoto, S. Kim, M. Eto et al., "Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery," *American Journal of Cardiology*, vol. 88, no. 12, pp. 1457–1460, 2001.

[336] V. B. Schini-Kerth, C. Auger, J.-H. Kim, N. Etienne-Selloum, and T. Chataigneau, "Nutritional improvement of the endothelial control of vascular tone by polyphenols: role of NO and EDHF," *Pflügers Archiv*, vol. 459, no. 6, pp. 853–862, 2010.

[337] M. A. B. Rosales, K. C. Silva, J. B. L. de Faria, and J. M. L. de Faria, "Exogenous sod mimetic tempol ameliorates the early retinal changes reestablishing the redox status in diabetic hypertensive rats," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 8, pp. 4327–4336, 2010.

[338] C. G. Schnackenberg and C. S. Wilcox, "The SOD mimetic tempol restores vasodilation in afferent arterioles of experimental diabetes," *Kidney International*, vol. 59, no. 5, pp. 1859–1864, 2001.

[339] D. S. Weber and K. K. Griendling, "The Yin/Yang of superoxide dismutase mimetics: potential cardiovascular therapies?" *British Journal of Pharmacology*, vol. 139, no. 6, pp. 1059–1060, 2003.

[340] J. A. Rodríguez, A. Grau, E. Eguinoa et al., "Dietary supplementation with vitamins C and E prevents downregulation of endothelial NOS expression in hypercholesterolemia in vivo and in vitro," *Atherosclerosis*, vol. 165, no. 1, pp. 33–40, 2002.

[341] J.-H. Bae, E. Bassenge, K.-B. Kim et al., "Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress," *Atherosclerosis*, vol. 155, no. 2, pp. 517–523, 2001.

[342] R. Maio, M. Perticone, A. Sciacqua et al., "Oxidative stress impairs endothelial function in nondipper hypertensive patients," *Cardiovascular Therapeutics*, vol. 30, no. 2, pp. 85–92, 2012.

[343] L. V. d'Uscio, S. Milstien, D. Richardson, L. Smith, and Z. S. Katusic, "Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity," *Circulation Research*, vol. 92, no. 1, pp. 88–95, 2003.

[344] A. R. Crecelius, B. S. Kirby, W. F. Voyles, and F. A. Dinenno, "Nitric oxide, but not vasodilating prostaglandins, contributes to the improvement of exercise hyperemia via ascorbic acid in healthy older adults," *American Journal of Physiology*, vol. 299, no. 5, pp. H1633–H1641, 2010.

[345] J. D. Krier, E. L. Ritman, Z. Bajzer, J. C. Romero, A. Lerman, and L. O. Lerman, "Noninvasive measurement of concurrent single-kidney perfusion, glomerular filtration, and tubular function," *American Journal of Physiology*, vol. 281, no. 4, pp. F630–F638, 2001.

[346] J. Neužil, S. R. Thomas, and R. Stocker, "Requirement for, promotion, or inhibition by α-tocopherol of radical- induced initiation of plasma lipoprotein lipid peroxidation," *Free Radical Biology and Medicine*, vol. 22, no. 1-2, pp. 57–71, 1996.

[347] J. F. Keaney Jr., J. M. Gaziano, A. Xu et al., "Low-dose α-tocopherol improves and high-dose α-tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits," *Journal of Clinical Investigation*, vol. 93, no. 2, pp. 844–851, 1994.

[348] D. Versari, E. Daghini, M. Rodriguez-Porcel et al., "Chronic antioxidant supplementation impairs coronary endothelial function and myocardial perfusion in normal pigs," *Hypertension*, vol. 47, no. 3, pp. 475–481, 2006.

[349] E. Daghini, X.-Y. Zhu, D. Versari et al., "Antioxidant vitamins induce angiogenesis in the normal pig kidney," *American Journal of Physiology*, vol. 293, no. 1, pp. F371–F381, 2007.

[350] S. R. J. Maxwell, "Coronary artery disease—free radical damage, antioxidant protection and the role of homocysteine," *Basic Research in Cardiology*, vol. 95, supplement 1, pp. I65–I71, 2000.

[351] R. J. Reiter, D.-X. Tan, S. D. Paredes, and L. Fuentes-Broto, "Beneficial effects of melatonin in cardiovascular disease," *Annals of Medicine*, vol. 42, no. 4, pp. 279–285, 2010.

[352] R. Hardeland, D.-X. Tan, and R. J. Reiter, "Kynuramines, metabolites of melatonin and other indoles: the resurrection of an almost forgotten class of biogenic amines," *Journal of Pineal Research*, vol. 47, no. 2, pp. 109–126, 2009.

[353] G. Paradies, G. Petrosillo, V. Paradies, R. J. Reiter, and F. M. Ruggiero, "Melatonin, cardiolipin and mitochondrial bioenergetics in health and disease," *Journal of Pineal Research*, vol. 48, no. 4, pp. 297–310, 2010.

[354] R. J. Reiter, D.-X. Tan, and A. Korkmaz, "The circadian melatonin rhythm and its modulation: possible impact on hypertension," *Journal of Hypertension*, vol. 27, supplement 6, pp. S17–S20, 2009.

[355] A. Dominguez-Rodriguez, P. Abreu-Gonzalez, J. J. Sanchez-Sanchez, J. C. Kaski, and R. J. Reiter, "Melatonin and circadian biology in human cardiovascular disease," *Journal of Pineal Research*, vol. 49, no. 1, pp. 14–22, 2010.

[356] L. Paulis, O. Pechanova, J. Zicha et al., "Melatonin improves the restoration of endothelium-derived constricting factor signalling and inner diameter in the rat femoral artery after cessation of L-NAME treatment," *Journal of Hypertension*, vol. 28, supplement 1, pp. S19–S24, 2010.

[357] F. Simko and L. Paulis, "Melatonin as a potential antihypertensive treatment," *Journal of Pineal Research*, vol. 42, no. 4, pp. 319–322, 2007.

[358] C. F. Reyes-Toso, L. M. Linares, C. R. Ricci et al., "Melatonin restores endothelium-dependent relaxation in aortic rings of pancreatectomized rats," *Journal of Pineal Research*, vol. 39, no. 4, pp. 386–391, 2005.

[359] C. F. Reyes-Toso, D. Obaya-Naredo, C. R. Ricci et al., "Effect of melatonin on vascular responses in aortic rings of aging rats," *Experimental Gerontology*, vol. 42, no. 4, pp. 337–342, 2007.

[360] M. M. Anwar, A.-R. M. A. Meki, and H. H. Abu Rahma, "Inhibitory effects of melatonin on vascular reactivity: possible role of vasoactive mediators," *Comparative Biochemistry and Physiology C*, vol. 130, no. 3, pp. 357–367, 2001.

[361] M. L. Pita, M. Hoyos, I. Martin-Lacave, C. Osuna, J. M. Fernández-Santos, and J. M. Guerrero, "Long-term melatonin administration increases polyunsaturated fatty acid percentage in plasma lipids of hypercholesterolemic rats," *Journal of Pineal Research*, vol. 32, no. 3, pp. 179–186, 2002.

[362] S. Tengattini, R. J. Reiter, D.-X. Tan, M. P. Terron, L. F. Rodella, and R. Rezzani, "Cardiovascular diseases: protective effects of melatonin," *Journal of Pineal Research*, vol. 44, no. 1, pp. 16–25, 2008.

[363] H. Girouard and J. de Champlain, "Inhibitory effect of melatonin on α1-adrenergic-induced vasoconstriction in mesenteric beds of spontaneously hypertensive rats," *American Journal of Hypertension*, vol. 17, no. 4, pp. 339–346, 2004.

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Research Article

Nitric Oxide and Superoxide Anion Balance in Rats Exposed to Chronic and Long Term Intermittent Hypoxia

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Work at high altitude in shifts exposes humans to a new form of chronic intermittent hypoxia, with still unknown health consequences. We have established a rat model resembling this situation, which develops a milder form of right ventricular hypertrophy and pulmonary artery remodelling compared to continuous chronic exposure. We aimed to compare the alterations in pulmonary artery nitric oxide (NO) availability induced by these forms of hypoxia and the mechanisms implicated. Rats were exposed for 46 days to normoxia or hypobaric hypoxia, either continuous (CH) or intermittent (2 day shifts, CIH2x2), and assessed: NO and superoxide anion availability (fluorescent indicators and confocal microscopy); expression of phosphorylated endothelial NO synthase (eNOS), NADPH-oxidase (p22phox), and 3-nitrotyrosine (western blotting); and NADPH-oxidase location (immunohistochemistry). Compared to normoxia, (1) NO availability was reduced and superoxide anion was increased in both hypoxic groups, with a larger effect in CH, (2) eNOS expression was only reduced in CH, (3) NADPH-oxidase was similarly increased in both hypoxic groups, and (4) 3-nitrotyrosine was increased to a larger extent in CH. In conclusion, intermittent hypoxia reduces NO availability through superoxide anion destruction, without reducing its synthesis, while continuous hypoxia affects both, producing larger nitrosative damage which could be related to the more severe cardiovascular alterations.

1. Introduction

Exposure to hypoxia, in either chronic or intermittent conditions, is associated with cardiovascular alterations. Hypoxia-related diseases are well characterized in people living at high altitude (chronic hypoxia, CH) [1] and in obstructive sleep apnea (OSA), where hypoxic conditions are maintained intermittently for brief periods [2]. Another mode of intermittent hypoxia has arisen as a result of the recent settlements of mines and other activities at high altitude (chronic intermittent hypoxia, CIH), where subjects repeatedly ascend from sea level to 3800–4200 m and work in shifts, being exposed to hypoxia for longer periods than in OSA conditions [3]. The large number of workers under these circumstances, together with the known deleterious cardiovascular effects reported in OSA patients [4], makes it of utmost importance to study the mechanisms implicated in CIH alterations. Since this condition is a relatively recent phenomenon, studies are still limited and animal models are valuable. We have used an experimental model of CIH, where rats are exposed to hypobaric hypoxia in shifts resembling the human situation [5]. We and others have found that under these experimental conditions the rats develop pulmonary hypertension, pulmonary vascular remodeling, and right

ventricular hypertrophy [6, 7], which are milder compared to rats chronically exposed to hypoxia [8].

Hypoxia-induced pulmonary hypertension and right ventricular hypertrophy are related to pulmonary artery vasoconstriction and vascular remodeling [9, 10], endothelial nitric oxide (NO) being an important modulator of these responses [11, 12]. Human and animal studies demonstrate that OSA is associated with reduced NO availability, due to a decreased production or destruction by an excess of reactive oxygen species (ROS) [2] and pulmonary artery vasoconstriction has been suggested to result from imbalance between endothelial vasodilator factors and ROS [13]. NO-ROS misbalance can also contribute to remodeling process in the pulmonary vasculature, through modification of cell migration, proliferation, dedifferentiation, and apoptosis [14]. We have previously demonstrated that rats exposed to long term CIH exhibit pulmonary artery remodeling with characteristic features which differ from those found under chronic exposure conditions [15, 16]. While there are several studies in OSA conditions, to the best of our knowledge, there are no data regarding the effect of hypoxia maintained intermittently during days on NO/ROS balance in the pulmonary vasculature. Therefore, we aimed to analyze the possible alterations under this condition and to compare them with those induced by continuous hypoxic exposure. We have used a rat model mimicking the conditions of subjects working in shifts or permanently living at high altitude.

2. Materials and Methods

2.1. Animal Model. Three-month-old male Wistar rats were used. The rats were exposed to 22 ± 2°C, 12 h light/dark cycle and were maintained in separate cages, with food made available to them (20 g pellets/day per rat) and water *ad libitum*. Standard veterinary care was used during all of the experiments following institutional protocols for the study of animals and the procedures used were approved by the Institutional Research Ethics Committee of Universidad Arturo Prat (Chile). The rats were randomly assigned to one of the following groups:

normoxia control group (NX; $n = 10$);

chronic intermittent hypoxia, 2 days under hypobaric hypoxia and 2 days under normobaric normoxia for 46 days (CIH2x2; $n = 10$);

chronic hypoxia, continuous hypobaric hypoxia for 46 days (CH; $n = 10$).

Hypobaric hypoxia was simulated at Universidad Arturo Prat facilities in a hypobaric chamber at 428 Torr, which is equivalent to an altitude of 4600 m (PO_2 = 89 mmHg, PCO_2 = 0.15 mmHg, temperature 22 ± 2°C, and humidity 35 ± 5%). Control (sea level) conditions were PO_2 = 159 mmHg, PCO_2 = 0.29 mmHg, temperature 22 ± 2°C, and humidity 35 ± 5%. The animal model for chronic intermittent hypoxia exposure (CIH2x2) has been described previously [5, 8, 17]. This model involves 2 days of hypoxia and 2 days of normoxia over a period of 46 days. We maintained the rats for 46 days, since previous data indicate that the hematological and cardiovascular effects in the rat are maximal between 30 and 45 days of exposure to hypoxia [5]. The 2x2 regimen was chosen in order to mimic long term exposure to intermittent hypoxic conditions as experienced by human subjects working in shifts at high altitude, which is in the order of days (usually 7–14) [18], and including at least one full circadian cycle. The control group was placed in the same room at sea level during the 46-day period.

All the rats were weighted at day 1 and at day 46 using an Acculab V-1200 electronic balance. At day 46 a blood sample was taken from the tail and hematocrit was measured using a microcentrifuge (Eppendorf AG, Hamburg, Germany). Thereafter, the animals were euthanized with an overdose of anesthesia (Ketamine, 8 mg, i.p.) and the heart and the lungs were removed in a block for further dissection.

The heart was cut down and the right ventricle was detached from the heart, leaving *in situ* the septum portion together with the left ventricle. Both ventricles were weighed in an analytic balance (Acculab V-1200, Illinois, USA) and the ratio between the right ventricle/left ventricle plus the septum weight was used to measure the grade of right ventricular hypertrophy. We did not use total heart weight or right ventricular weight/body weight since we have previously reported that exposure to hypoxia produces alterations in body weight gain [5]. The removed lung was placed in a Petri dish and pulmonary artery branches (4th order) were dissected and stored for further experiments in saline solution (0.9% NaCl).

2.2. Confocal Microscopy

2.2.1. Determination of NO Availability. Basal NO availability was determined by the fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2 DA, Sigma) as described previously [19]. Briefly, 3 mm length pulmonary artery segments were stabilized in physiological salt solution (PSS; 115 mmol/L NaCl, 4.6 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L, $MgSO_4$, and 2.5 mmol/L $CaCl_2$), for 30 min at 37°C, and oxygenated with carbogen (95% O_2 and 5% CO_2). Thereafter, they were stained with oxygenated DAF-2 DA solution (10 μmol/L) for 30 min in the darkness at 37°C, in a shaking water bath. This experimental procedure in saturated oxygen ensures that NO, rather than O_2, is the limiting factor in the reaction and the fluorescence is directly proportional to NO [19]. Negative controls for DAF-2 DA were incubated in 0.1 mmol/L L-NAME throughout the experimental period. The segments were then washed 3 times for 1 min each in PSS and fixed in 4% (w/v) paraformaldehyde. The segments were cut in rings with a blade and mounted on a slide equipped with a small well made of spacers, filled with mounting medium (Citifluor, Aname, Spain), and covered with a cover glass. The arterial rings were visualized with a Leica TCS SP2 confocal system (Leica Microsystems, Wetzlar, Germany) at Universidad Autónoma de Madrid of Spain facilities using the 488 nm/515 nm line. 1 μm thick serial images (25 μm in total) were captured with a 63x objective at zoom 2 in 3

randomly chosen areas of the ring, at identical conditions of brightness, contrast, and laser power for all of the experimental groups. MetaMorph image analysis software (Universal Imaging Co., UK) was used for quantification of fluorescence intensity. Briefly, the serial images were first reconstructed in a confocal projection and fluorescence intensity was quantified in several regions of the smooth muscle cells, where the dye is trapped, avoiding the elastic lamella which is also fluorescent in the same wavelength.

2.2.2. Determination of $O_2^{\bullet-}$ Availability. Dihydroethidium (DHE, Sigma) was used to determine basal $O_2^{\bullet-}$, as described [19]. Briefly, 3 mm long pulmonary arteries were stabilized in PSS (30 min at 37°C). Thereafter, they were incubated with 3 μmol/L DHE, washed 3 times for 1 min each in PSS, and fixed in 4% (w/v) paraformaldehyde. Negative controls for DHE were incubated in 15 units/mL superoxide dismutase (SOD) throughout the incubation period. The segments were then washed 3 times for 1 min each in PSS, fixed in 4% (w/v) paraformaldehyde, cut in rings, and mounted as described above. 1 μm thick serial images (25 μm in total) were captured with a 63x objective at zoom 2 in 3 randomly chosen areas of the ring, at identical conditions of brightness, contrast, and laser power for all of the experimental groups with the 488 nm/590–620 nm line of the microscope. MetaMorph image analysis software (Universal Imaging Co., UK) was used for quantification of fluorescence intensity, which was located in the nuclei. The serial images were first reconstructed in a confocal projection and fluorescence intensity was quantified in several regions along the ring.

2.2.3. NADPH-Oxidase Detection by Immunohistochemistry. To detect the presence of NADPH oxidase in the adventitial layer, pulmonary arteries were first incubated with the primary antibody of the p22phox subunit of the enzyme (rabbit polyclonal, Santa Cruz Biotechnology, USA) (60 min, 1 : 200 in saline solution) at room temperature (RT) and then washed with saline solution (30 min, RT). Thereafter, the segments were incubated with the secondary antibody-Alexa Fluor 647 goat anti-rabbit IgG (H + L) (Invitrogen, Madrid, Spain) (60 min, 1 : 200, RT in the darkness), followed by washing for 30 min, RT. Finally, they were incubated with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI; 1 : 500 from a 5 mg/mL stock; 30 min, RT in the darkness) and washed twice (30 min, RT). The pulmonary arteries were longitudinally sectioned and mounted with the adventitial side facing up, as described above. The arteries were visualized with a Leica TCS confocal system using the 405 nm excitation/410–475 nm emission wavelength (DAPI) to locate the cells and the 633 nm excitation/640–650 nm emission wavelength (secondary antibody-Alexa 647) to detect the protein. 1 μm thick serial images of the adventitial layer (12 μm in total) were captured at both wavelengths with a 40x objective at zoom 4 from 3 different regions. MetaMorph software was used to count the total number of cells (DAPI positive) and those stained with p22phox. Cell number (DAPI or p22phox positive) was counted always in the same volume which was calculated from the layer thickness (12 μm) and the image area at ×40 zoom 4. We calculated the total number of p22phox positive cells and the relative number of positive cells (positive cells/total cells) in the mentioned volume.

2.3. Western Blot. Western blot was performed according to the standard methods. Briefly, total pulmonary artery tissues were frozen in liquid nitrogen and then homogenized into homogenization buffer containing 50 mM Tris-HCl, 150 mM NaCl, 100 mM NaF, 1% Triton X-100, 1 mM dithiothreitol, 0.1 mM phenyl methylsulfonyl fluoride, 1 mM leupeptin, 0.02 M Hepes, 10^{-3} M EDTA, 10^{-3} M EGTA, and 20% glycerol, followed by centrifugation to 10000 ×g by 10 min. Supernatant was removed and protein quantification was carried out by Bradford assay. Equal amount of protein (25 μg) was resolved on 7.5–12% SDS-PAGE and proteins were transferred to a PVDF membrane. The nonspecific binding sites on the membrane were blocked using 5% nonfat dry milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% tween 20, and pH 7.4) by 1 hour. Membranes were incubated with primary antibody rabbit polyclonal to PNK (3-nitrotyrosine) and p22phox and goat polyclonal pNOS-3 (phosphorylated form of e-NOS; Santa Cruz Biotechnology, USA) by overnight and washed three times for 15, 10, 5 min with TBS-T followed by incubation with horseradish peroxidase-conjugated secondary antibody (1 : 2000, Santa Cruz Biotechnology, USA) for 1 hour and washed 3 times with TBS-T and once with TBS. Blots were visualized using a West Pico Chemiluminescence System (Pierce, USA) and then analyzed using Image J. Expression levels of p22phox, PNK, and pNOS3 were normalized to β-actin expression.

2.4. Statistical Analysis. The experimental data were entered into a database and were analyzed using SPSS 17.0 statistical package (SPSS, Inc., Chicago, Ill, USA). Mean, standard deviation, and standard error were calculated for each parameter. Normality was established using the Kolmogorov-Smirnov test. Statistical analysis of the differences across all testing conditions was established using analysis of variance (ANOVA) of one factor and less significant difference post hoc tests. All of the variables were normally distributed. Statistical significance was established at a P value < 0.05.

3. Results

3.1. Body Weight Gain, Hematocrit, and Right Ventricular Hypertrophy. Body weight at day 1 was not statistically different between experimental groups (NX: 247.8 ± 16.6 gr, CIH2x2: 246.9 ± 11.2 gr, and CH: 251.5 ± 17.2 gr). While NX rats gained weight over the 46-day period (final body weight NX = 330 ± 13.5 g), there was a gradual weight loss in both hypoxic groups (final body weight CIH2x2 = 206 ± 8.03 g; CH = 169 ± 3.6 g), being significantly smaller compared to NX ($P < 0.001$). Hematocrit at the end of experimental period was significantly higher in CH (66 ± 1.1%) and in CIH2x2 (58 ± 1.8%), compared to NX rats (51 ± 1.0%) ($P < 0.01$). CIH2x2 hematocrit was significantly smaller compared to CH ($P < 0.01$). Right ventricular weight/total heart weight

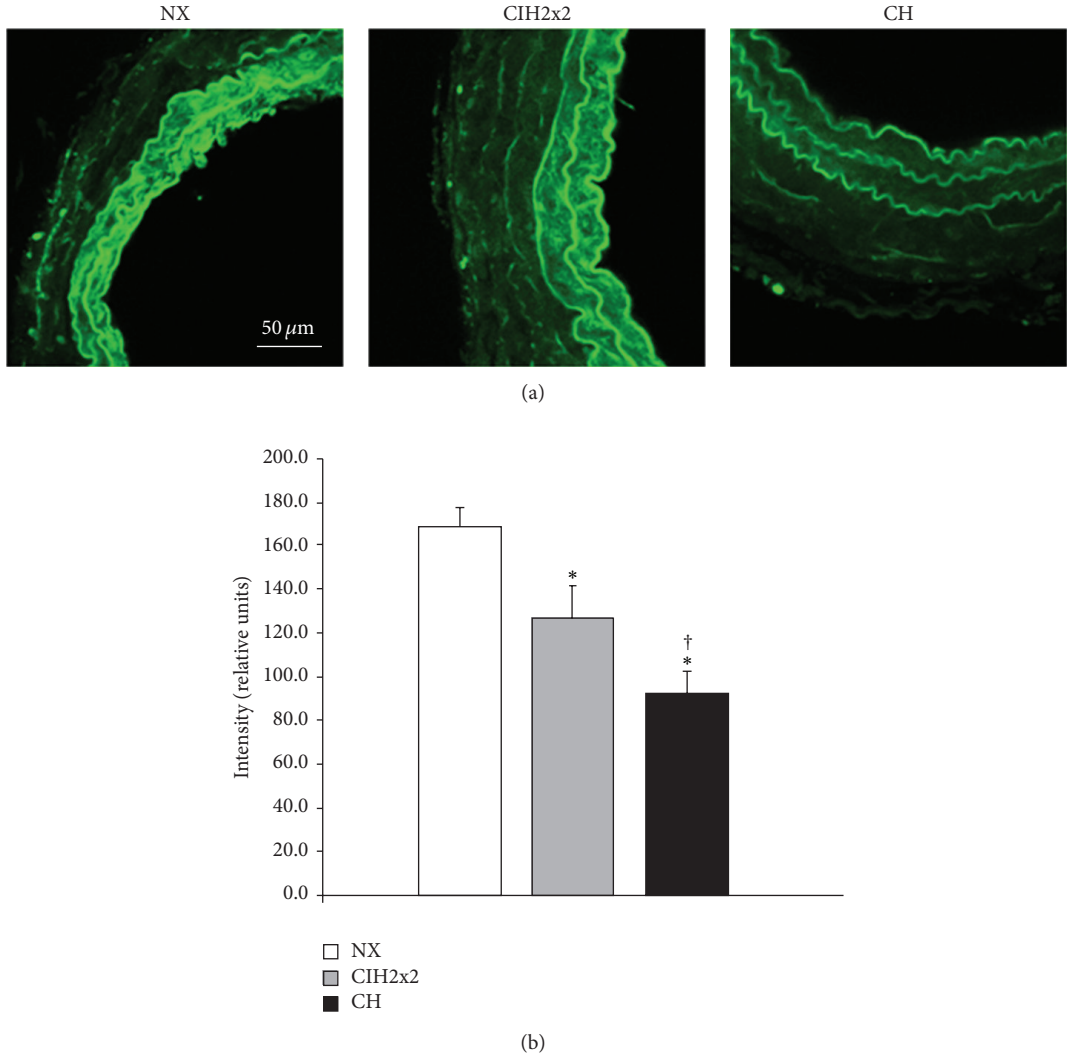

Figure 1: DAF-2 DA intensity levels in pulmonary arteries from rats exposed to normoxia (NX, $n = 10$), intermittent hypoxia (CIH2x2, $n = 10$), or chronic hypoxia (CH, $n = 10$). (a) Representative examples of projections obtained from confocal microscopy images (×40 zoom 2). (b) Quantitative analysis; n represents the number of animals; $^{*}P < 0.05$ compared to NX; $^{\dagger}P < 0.05$ compared to CIH2x2.

was smaller in NX (NX = 0.23 ± 0.02; $P < 0.01$) compared to CIH2x2 rats (0.34 ± 0.02) and CH group (0.40 ± 0.02), which was also significantly larger compared to intermittent exposure group ($P < 0.05$).

3.2. NO Availability. The fluorescence emitted by DAF-2 DA was located in the cytoplasm of smooth muscle cells. Pulmonary arteries from NX group exhibited a significantly higher DAF-2 DA emitted fluorescence compared to both hypoxic groups, suggesting a larger basal NO availability [19]. Fluorescence was significantly lower in CH rats compared to CIH2x2 (Figure 1).

3.3. $O_2^{\bullet-}$ Availability. DHE fluorescence was located in the cell nuclei. NX rats showed a smaller level of fluorescence intensity compared to both hypoxic groups, being higher in CH compared to CIH2x2 (Figure 2).

3.4. Phosphorylated eNOS, p22phox, and 3-Nitrotyrosine Expression. Expression of the phosphorylated form of eNOS was significantly reduced in CH compared to NX. The expression levels in CIH2x2 were not statistically different from NX (Figure 3(a)).

p22phox expression was scarcely detectable in pulmonary arteries from NX but was largely increased in both hypoxic rats, without differences between CH and CIH2x2 groups (Figure 3(b)).

3-Nitrotyrosine expression, a marker of nitrosative damage, was significantly elevated in pulmonary arteries from hypoxic rats compared to NX, being significantly larger in CH compared to CIH2x2 (Figure 3(c)).

3.5. p22phox Location in the Adventitia. The total number of adventitial cells, quantified by the nuclear dye DAPI in a fixed volume, was significantly higher in pulmonary arteries from

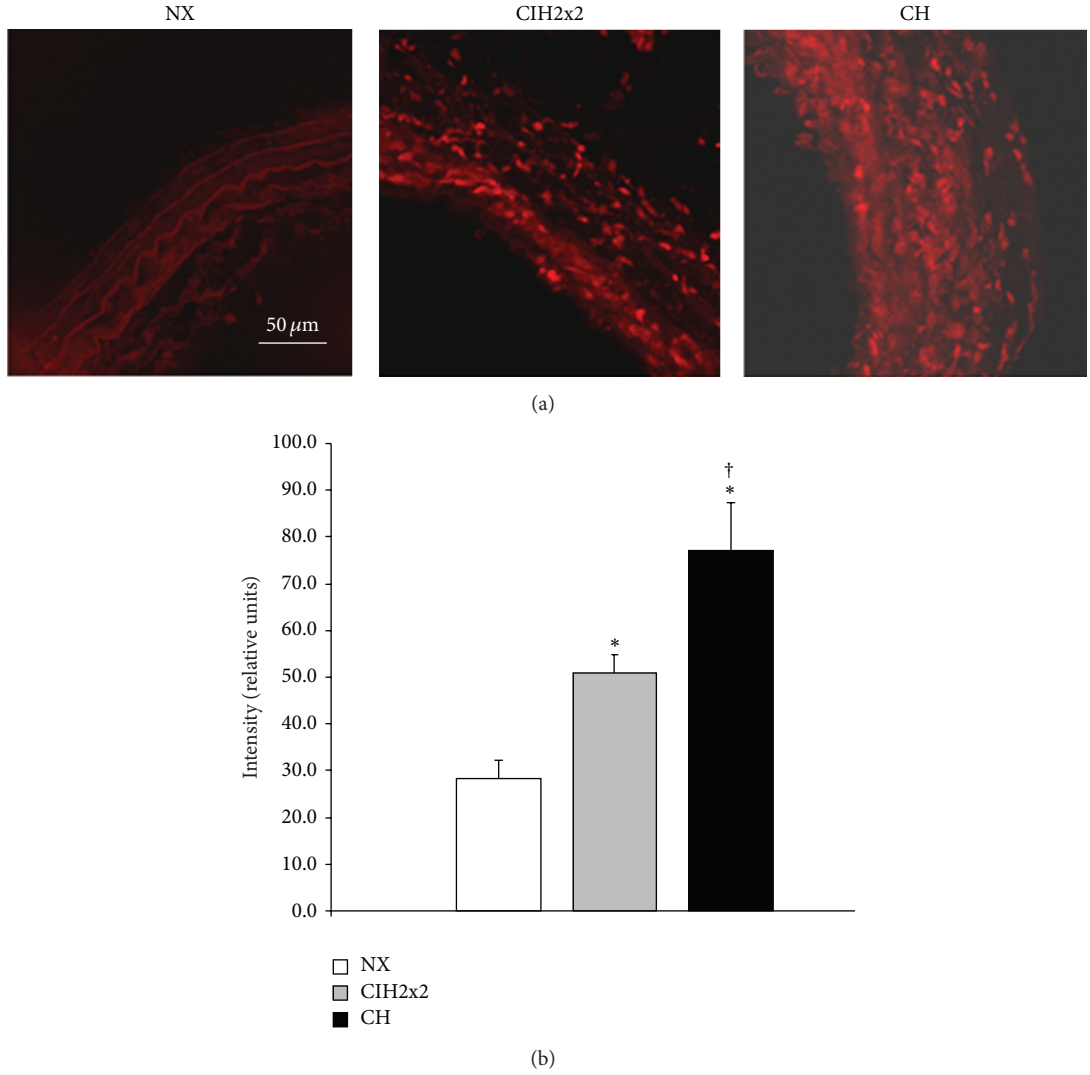

FIGURE 2: DHE intensity levels in pulmonary arteries from rats exposed to normoxia (NX, $n = 10$), intermittent hypoxia (CIH2x2, $n = 10$), or chronic hypoxia (CH, $n = 10$). (a) Representative examples of projections obtained from confocal microscopy images (×40 zoom 2). (b) Quantitative analysis; n represents the number of animals; $^{*}P < 0.05$ compared to NX; $^{\dagger}P < 0.05$ compared to CIH2x2.

both hypoxic rats (CIH2x2 = 144.7 ± 13.2; CH = 157.1 ± 9.7) compared to control (NX = 74.9 ± 2.6, $P < 0.001$).

P22phox staining was observed in the three experimental groups and was located around some of the adventitial cells. Pulmonary arteries from hypoxic rats exhibited a larger level of staining compared to NX (Figure 4(a)). Both total and relative numbers of p22phox positive cells were significantly larger in the adventitial layer of hypoxic rats compared to NX, with no statistical difference between CIH2x2 and CH rats (Figure 4(b)).

4. Discussion

The main findings of the current study are that exposure to chronic intermittent hypoxia reduces NO availability in the pulmonary vasculature. This decrease is likely due to NO destruction by $O_2^{\bullet -}$, generated by NADPH-oxidase, while eNOS is not altered. On the other hand, in continuous exposure to hypoxia, NO availability is further reduced, through the combination of diminished NO synthesis and increased destruction. In consequence, chronic hypoxia produces larger nitrosative damage compared to intermittent exposure, which likely contributes to the higher impact on pulmonary artery remodeling and right ventricular hypertrophy (Figure 5 summarizes these results).

The present data confirmed that hypoxia induced weight loss, right ventricular hypertrophy, and hematocrit increase, as previously described by us [8] and others [6, 7]. We have also described remodeling of the pulmonary vasculature [15, 16], these alterations being less severe under intermittent exposure. We aimed to assess if the above mentioned cardiovascular alterations are linked to NO/ROS misbalance. To the best of our knowledge, there is virtually no information in long term intermittent hypoxia conditions, the majority of evidence coming from OSA studies [20], where low oxygen levels are maintained for very brief periods.

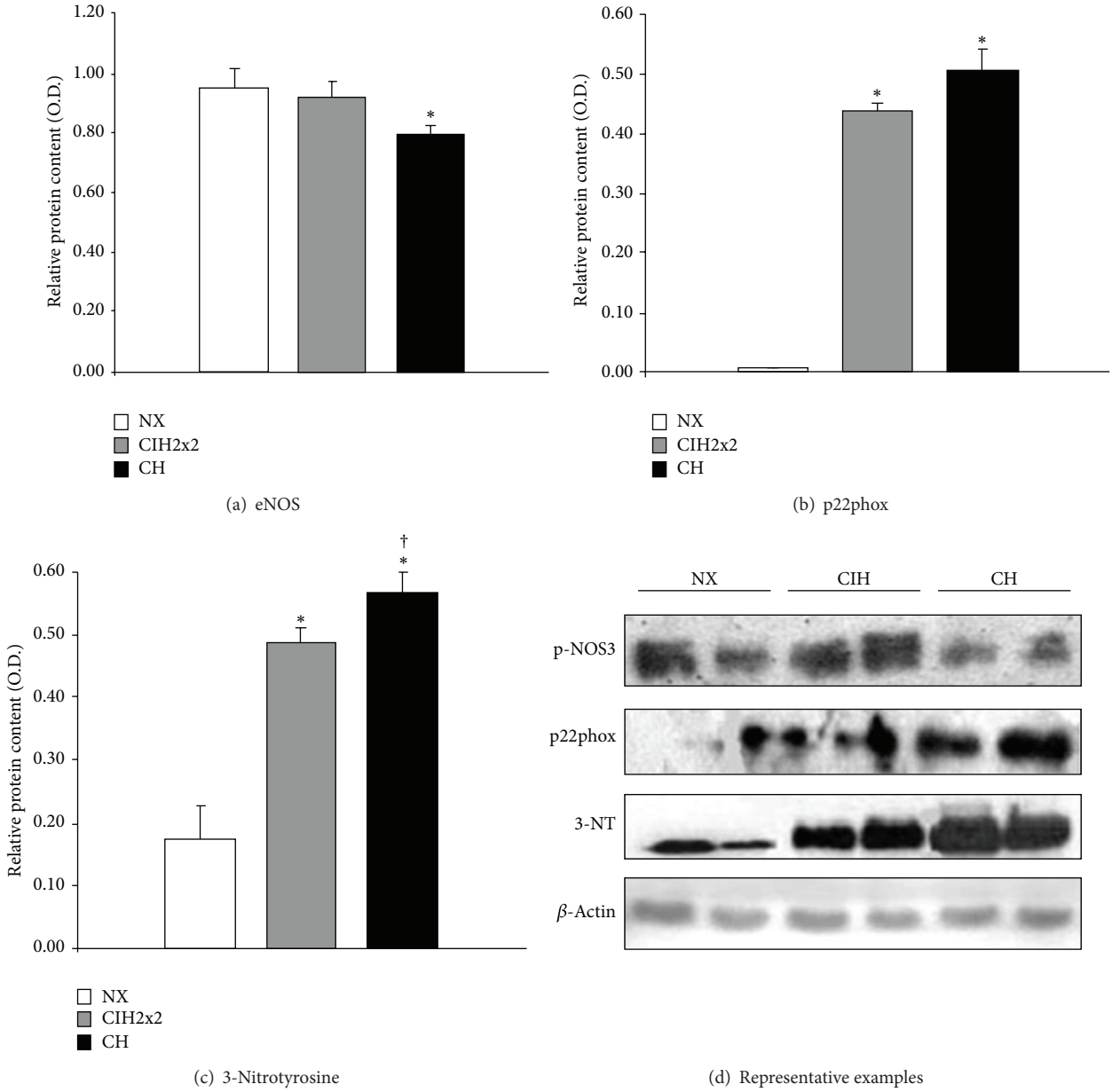

Figure 3: Western blot analysis of: (a) phosphorylated eNOS, (b) p22phox, and (c) 3-nitrotyrosine from pulmonary arteries of rats exposed to normoxia (NX, $n = 9$), intermittent hypoxia (CIH2x2, $n = 10$), or chronic hypoxia (CH, $n = 10$). (d) Representative examples. n represents the number of animals; $^{*}P < 0.05$ compared to NX; $^{\dagger}P < 0.05$ compared to CIH2x2.

We focused on NO, a key factor for pulmonary artery resistance [12], since diminished NO availability likely affects both pulmonary artery structure and function. Furthermore, studies on rodents have revealed that intermittent exposure to hypoxia is associated with NO reduction in the systemic and cerebral vasculature [21, 22]. Decreased NO availability in the pulmonary vasculature can be the result of a reduced production by eNOS and/or an increased destruction by ROS, particularly $O_2^{\bullet-}$, which has been implicated in hypoxic pulmonary vasoconstriction [13, 23–25]. To determine NO availability, we used DAF-2 DA, a fluorescent indicator directly proportional to the amount of NO [26, 27]. Using confocal microscopy and image analysis software, we have previously demonstrated that this method is sufficiently sensitive for the quantification of basal NO in resistance and conduit arteries [19, 28, 29]. Continuous hypoxia induced a larger reduction of NO availability, compared to intermittent exposure. This can be explained by a reduced NO production, as suggested by the decreased expression of phosphorylated eNOS—the active form of the enzyme—found in chronic hypoxia only. The lack of effect of intermittent exposure could be explained by the functional "on-off" (hypoxia-normoxia) switch [30] which is not able to induce downregulation of the NO biosynthetic machinery.

Reduced NO availability in hypoxic conditions seems also to be related to NO destruction by $O_2^{\bullet-}$. This is

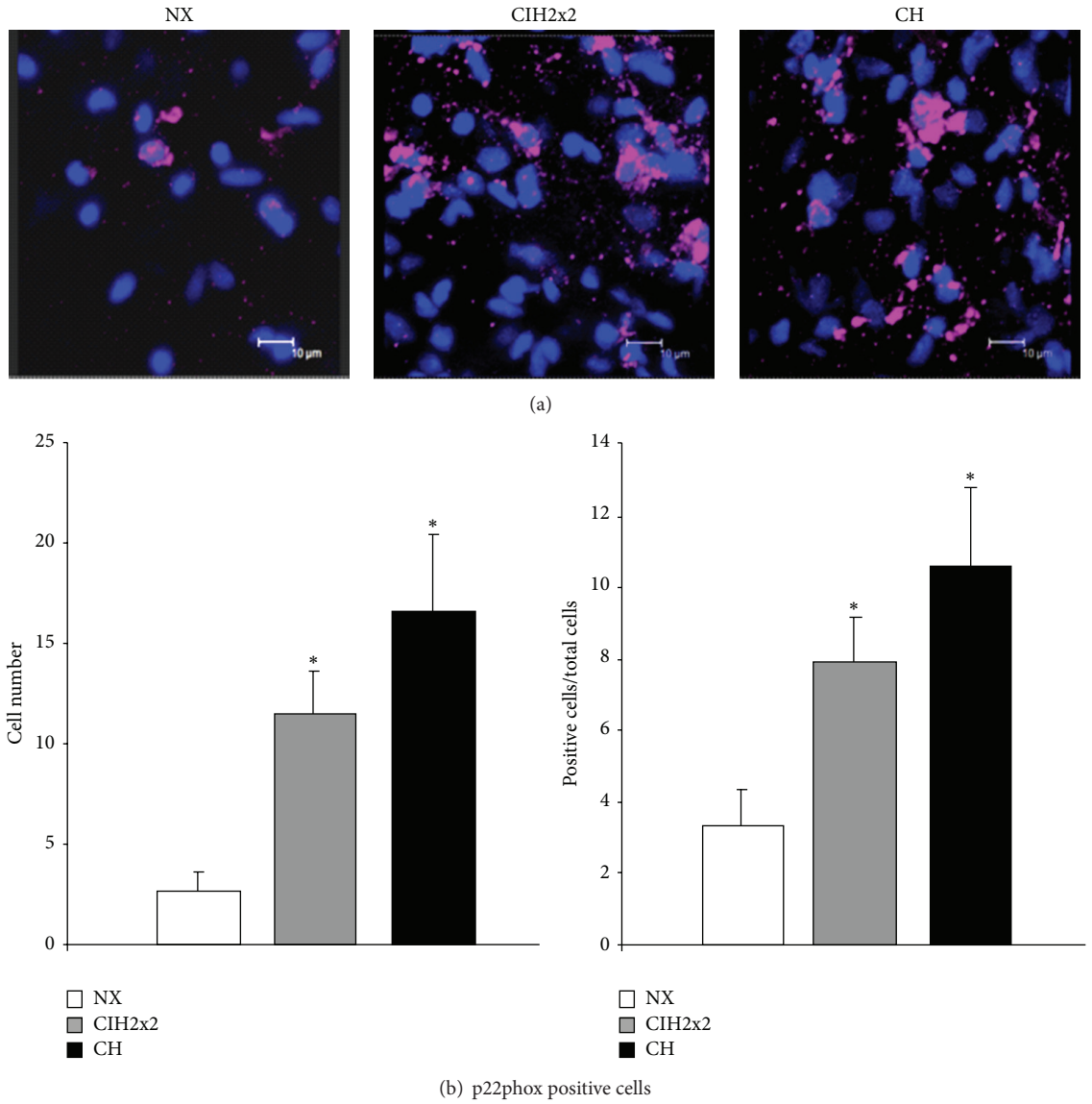

FIGURE 4: Immunohistochemical detection of p22phox positive cells in the adventitia of pulmonary arteries from rats exposed to normoxia (NX, $n = 10$), intermittent hypoxia (CIH2x2, $n = 10$), or chronic hypoxia (CH, $n = 10$). (a) Representative examples of projections obtained from confocal microscopy images (×40 zoom 4). (b) Quantitative analysis; n represents the number of animals; $^{*}P < 0.05$ compared to NX.

suggested by the increased superoxide anion production found in CIH2x2 and CH pulmonary arteries, similarly to data described in an OSA rat model [31]. Superoxide anion levels were even higher in continuous compared to intermittent hypoxia, suggesting that continuous exposure further stimulates the enzymatic systems responsible for ROS synthesis. In the vascular wall, several enzymes produce $O_2^{\bullet-}$, NADPH oxidase being the main system in the pulmonary vasculature [13, 32]. We found a remarkable expression of this enzyme—confirmed by immunohistochemistry—in both CH and CIH2x2, while it was very low in normoxic rats. The important role of this enzyme in intermittent hypoxia has been previously demonstrated in NADPH-oxidase knock-out mice exposed to OSA [33]. Our data show that $O_2^{\bullet-}$ levels were larger in continuous compared to intermittent hypoxia, despite the similar p22phox expression in both groups. This could be explained by $O_2^{\bullet-}$ production by xanthine oxidase, as suggested in OSA patients and animal models [34, 35]. Since it has been reported that, under hypoxic conditions, $O_2^{\bullet-}$ generated by xanthine oxidase is very small [36], alternatively, $O_2^{\bullet-}$ can be produced by "uncoupled" dysfunctional eNOS, which can be induced by peroxynitrite [37]. We did not measure peroxynitrite directly but quantified 3-nitrotyrosine, which is currently accepted as evidence of peroxynitrite generation. The larger content of 3-nitrotyrosine found in CH suggests that eNOS uncoupling can contribute to larger $O_2^{\bullet-}$ production found in continuous hypoxic conditions compared to intermittent conditions, despite the similar NADPH oxidase expression in both groups.

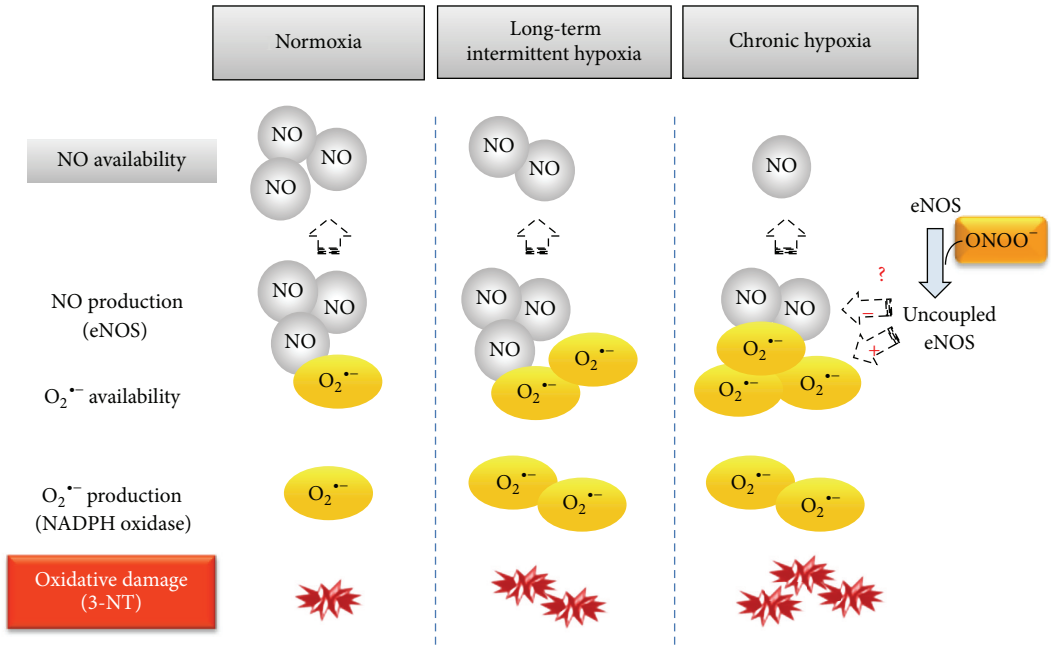

FIGURE 5: Schematic diagram showing the main results and the proposed mechanism implicated in the $NO/O_2^{\bullet-}$ misbalance induced by chronic and intermittent hypoxia.

Oxidative-nitrosative stress is associated with vascular remodeling in hypoxia-induced pulmonary hypertension [38]. We have previously found several signs of remodeling in pulmonary arteries from CH and CIH2x2 rats, including wall hypertrophy due to increased smooth muscle and adventitial cells [15, 16]. Adventitial NADPH oxidase has been described to be involved in pulmonary artery adventitial fibroblasts proliferation [39, 40] and seems to be a primary site of superoxide anion production in the vessel wall [41–43]. We found that chronic or intermittent hypoxia substantially increased adventitial cell number together with a larger percentage of NADPH-positive cells. Moreover, we [44] and others [43] have previously demonstrated that adventitia is a key layer regarding NO inactivation by ROS. Since NO is an antiproliferative agent, we suggest that an imbalance between $NO/O_2^{\bullet-}$ can be linked to the vascular remodeling process under continuous or intermittent hypoxic exposure.

It was beyond the scope of this study to investigate the mechanisms implicated in hypoxia-induced NO/ROS disbalance. However, we can speculate on the possible role of inflammation. Alveolar hypoxia produces widespread systemic inflammation [45] and it also promotes the development of a pulmonary artery chronic inflammatory microenvironment [46]. We also have evidence of infiltrated macrophages in pulmonary arteries from CH and CIH2x2 (unpublished results), suggesting that local inflammation in the vascular wall might contribute to the NO/ROS disbalance. In support of this hypothesis, there is evidence that tumor necrosis factor-α can reduce eNOS expression and activity in pulmonary arteries [47]. Moreover, inflammation associated with macrophage infiltration can also contribute to ROS generation through stimulation of NADPH oxidase expression, as previously found in the carotid body under intermittent hypoxic conditions [48].

In conclusion, the present study suggests that hypobaric hypoxia under intermittent conditions reduces NO availability due to destruction by superoxide anion, without affecting NO synthesis, while continuous exposure is associated with both increased degradation and reduced NO production. The oxidative-nitrosative stress induced by long term intermittent hypoxia might participate in the observed cardiovascular structural alterations but represents a milder form of damage compared to continuous exposure. These data suggest that the alterations in oxidative status of humans chronically or intermittently exposed to high altitude need to be evaluated, in order to improve the associated cardiovascular alterations.

Conflict of Interests

There is no conflict of interests.

Funding

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References

[1] F. León-Velarde, M. Maggiorini, J. T. Reeves et al., "Consensus statement on chronic and subacute high altitude diseases," *High Altitude Medicine and Biology*, vol. 6, no. 2, pp. 147–157, 2005.

[2] R. Dumitrascu, J. Heitmann, W. Seeger, N. Weissmann, and R. Schulz, "Obstructive sleep apnea, oxidative stress and cardiovascular disease: lessons from animal studies," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 234631, 7 pages, 2013.

[3] J.-P. Richalet, M. V. Donoso, D. Jiménez et al., "Chilean miners commuting from sea level to 4500 m: a prospective study," *High Altitude Medicine and Biology*, vol. 3, no. 2, pp. 159–166, 2002.

[4] G. E. Foster, J. V. Brugniaux, V. Pialoux et al., "Cardiovascular and cerebrovascular responses to acute hypoxia following exposure to intermittent hypoxia in healthy humans," *Journal of Physiology*, vol. 587, no. 13, pp. 3287–3299, 2009.

[5] P. Siqués Lee, J. Brito, F. León-Velarde et al., "Time course of cardiovascular and hematological responses in rats exposed to chronic intermittent hypobaric hypoxia (4600 m)," *High Altitude Medicine and Biology*, vol. 7, no. 1, pp. 72–80, 2006.

[6] A. F. Corno, G. Milano, S. Morel et al., "Hypoxia: unique myocardial morphology?" *Journal of Thoracic and Cardiovascular Surgery*, vol. 127, no. 5, pp. 1301–1308, 2004.

[7] M. McGuire and A. Bradford, "Chronic intermittent hypoxia increases haematocrit and causes right ventricular hypertrophy in the rat," *Respiration Physiology*, vol. 117, no. 1, pp. 53–58, 1999.

[8] J. Brito, P. Siqués, F. León-Velarde et al., "Varying exposure regimes to long term chronic intermittent hypoxia exert different outcomes and morphological effects on Wistar rats at 4600 m," *Toxicological and Environmental Chemistry*, vol. 90, no. 1, pp. 169–179, 2008.

[9] A. G. Durmowicz and K. R. Stenmark, "Mechanisms of structural remodeling in chronic pulmonary hypertension," *Pediatrics in Review*, vol. 20, no. 11, pp. e91–e102, 1999.

[10] N. Sommer, A. Dietrich, R. T. Schermuly et al., "Regulation of hypoxic pulmonary vasoconstriction: basic mechanisms," *European Respiratory Journal*, vol. 32, no. 6, pp. 1639–1651, 2008.

[11] M. S. Wolin, S. A. Gupte, C. J. Mingone, B. H. Neo, Q. Gao, and M. Ahmad, "Redox regulation of responses to hypoxia and NO-cGMP signaling in pulmonary vascular pathophysiology," *Annals of the New York Academy of Sciences*, vol. 1203, pp. 126–132, 2010.

[12] W. Steudel, M. Scherrer-Crosbie, K. D. Bloch et al., "Sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia in mice with congenital deficiency of nitric oxide synthase," *Journal of Clinical Investigation*, vol. 101, no. 11, pp. 2468–2477, 1998.

[13] G. Frazziano, H. C. Champion, and P. J. Pagano, "NADPH oxidase-derived ROS and the regulation of pulmonary vessel tone," *The American Journal of Physiology*, vol. 302, no. 11, pp. H2166–H2177, 2012.

[14] K. K. Griendling and M. Ushio-Fukai, "Reactive oxygen species as mediators of angiotensin II signaling," *Regulatory Peptides*, vol. 91, no. 1–3, pp. 21–27, 2000.

[15] M. C. González, "Confocal Microscopy as New Tool for the Study of Pulmonary Artery Remodelling at a Cellular Level in Rats Exposed to Chronic Hypobaric Hypoxia," vol. 11, pp. 262, 2010.

[16] P. Siques, "Structural changes in pulmonary artery of exposed rats to chronic intermittent hypobaric hypoxia," *High Altitude Medicine and Biology*, vol. 11, article 290, 2010.

[17] R. Germack, F. Leon-Velarde, R. Valdes De La Barra, J. Farias, G. Soto, and J. P. Richalet, "Effect of intermittent hypoxia on cardiovascular function, adrenoceptors and muscarinic receptors in Wistar rats," *Experimental Physiology*, vol. 87, no. 4, pp. 453–460, 2002.

[18] J. Brito, P. Siqués, F. León-Velarde, J. J. De La Cruz, V. López, and R. Herruzo, "Chronic intermittent hypoxia at high altitude exposure for over 12 years: assessment of hematological, cardiovascular, and renal effects," *High Altitude Medicine and Biology*, vol. 8, no. 3, pp. 236–244, 2007.

[19] J. M. González, B. Somoza, M. V. Conde, M. S. Fernández-Alfonso, M. C. González, and S. M. Arribas, "Hypertension increases middle cerebral artery resting tone in spontaneously hypertensive rats: role of tonic vasoactive factor availability," *Clinical Science*, vol. 114, no. 9-10, pp. 651–659, 2008.

[20] Z. Wang, A. Y. Li, Q. H. Guo et al., "Effects of cyclic intermittent hypoxia on ET-1 responsiveness and endothelial dysfunction of pulmonary arteries in rats," *PLoS ONE*, vol. 8, no. 3, Article ID e58078, 2013.

[21] Z. Tahawi, N. Orolinova, I. G. Joshua, M. Bader, and E. C. Fletcher, "Altered vascular reactivity in arterioles of chronic intermittent hypoxic rats," *Journal of Applied Physiology*, vol. 90, no. 5, pp. 2007–2000, 2001.

[22] S. A. Phillips, E. B. Olson, B. J. Morgan, and J. H. Lombard, "Chronic intermittent hypoxia impairs endothelium-dependent dilation in rat cerebral and skeletal muscle resistance arteries," *The American Journal of Physiology*, vol. 286, no. 1, pp. H388–H393, 2004.

[23] D. C. Irwin, J. M. McCord, E. Nozik-Grayck et al., "A potential role for reactive oxygen species and the HIF-1α-VEGF pathway in hypoxia-induced pulmonary vascular leak," *Free Radical Biology and Medicine*, vol. 47, no. 1, pp. 55–61, 2009.

[24] N. Weissmann, R. T. Schermuly, H. A. Ghofrani et al., "Hypoxic pulmonary vasoconstriction: triggered by an increase in reactive oxygen species?" *Novartis Foundation Symposium*, vol. 272, pp. 196–208, 2006.

[25] N. Weissmann, S. Zeller, R. U. Schäfer et al., "Impact of mitochondria and NADPH oxidases on acute and sustained hypoxic pulmonary vasoconstriction," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 34, no. 4, pp. 505–513, 2006.

[26] H. Kojima, N. Nakatsubo, K. Kikuchi et al., "Direct evidence of NO production in rat hippocampus and cortex using a new fluorescent indicator: DAF-2 DA," *NeuroReport*, vol. 9, no. 15, pp. 3345–3348, 1998.

[27] F.-X. Yi, A. Y. Zhang, W. B. Campbell, A.-P. Zou, C. Van Breemen, and P.-L. Li, "Simultaneous in situ monitoring of intracellular Ca2+ and NO in endothelium of coronary arteries," *The American Journal of Physiology*, vol. 283, no. 6, pp. H2725–H2732, 2002.

[28] S. M. Arribas, C. J. Daly, M. C. González, and J. C. Mcgrath, "Imaging the vascular wall using confocal microscopy," *Journal of Physiology*, vol. 584, no. 1, pp. 5–9, 2007.

[29] B. Somoza, F. Abderrahim, J. M. González et al., "Short-term treatment of spontaneously hypertensive rats with liver growth factor reduces carotid artery fibrosis, improves vascular function, and lowers blood pressure," *Cardiovascular Research*, vol. 69, no. 3, pp. 764–771, 2006.

[30] F. L. Powell and N. Garcia, "Physiological effects of intermittent hypoxia," *High Altitude Medicine and Biology*, vol. 1, no. 2, pp. 125–136, 2000.

[31] C. E. Norton, N. L. Jernigan, N. L. Kanagy, B. R. Walker, and T. C. Resta, "Intermittent hypoxia augments pulmonary vascular smooth muscle reactivity to NO: regulation by reactive oxygen species," *Journal of Applied Physiology*, vol. 111, no. 4, pp. 980–988, 2011.

[32] B. Fuchs, N. Sommer, A. Dietrich et al., "Redox signaling and reactive oxygen species in hypoxic pulmonary vasoconstriction," *Respiratory Physiology and Neurobiology*, vol. 174, no. 3, pp. 282–291, 2010.

[33] R. E. Nisbet, A. S. Graves, D. J. Kleinhenz et al., "The role of NADPH oxidase in chronic intermittent hypoxia-induced pulmonary hypertension in mice," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 40, no. 5, pp. 601–609, 2009.

[34] J. M. Dopp, N. R. Philippi, N. J. Marcus et al., "Xanthine oxidase inhibition attenuates endothelial dysfunction caused by chronic intermittent hypoxia in rats," *Respiration*, vol. 82, no. 5, pp. 458–467, 2011.

[35] A. A. El Solh, R. Saliba, T. Bosinski, B. J. B. Grant, E. Berbary, and N. Miller, "Allopurinol improves endothelial function in sleep apnoea: a randomised controlled study," *European Respiratory Journal*, vol. 27, no. 5, pp. 997–1002, 2006.

[36] I. Al Ghouleh, N. K. H. Khoo, U. G. Knaus et al., "Oxidases and peroxidases in cardiovascular and lung disease: new concepts in reactive oxygen species signaling," *Free Radical Biology and Medicine*, vol. 51, no. 7, pp. 1271–1288, 2011.

[37] U. Förstermann, "Nitric oxide and oxidative stress in vascular disease," *Pflügers Archiv*, vol. 459, no. 6, pp. 923–939, 2010.

[38] S. Aggarwal, C. M. Gross, S. Sharma, J. R. Fineman, and S. M. Black, "Reactive oxygen species in pulmonary vascular remodeling," *Comprehensive Physiology*, vol. 3, no. 3, pp. 1011–1034.

[39] E. Panzhinskiy, W. M. Zawada, K. R. Stenmark, and M. Das, "Hypoxia induces unique proliferative response in adventitial fibroblasts by activating PDGFbeta receptor-JNK1 signalling," *Cardiovascular Research*, vol. 95, no. 3, pp. 356–365, 2012.

[40] S. Li, S. S. Tabar, V. Malec et al., "NOX4 regulates ROS levels under normoxic and hypoxic conditions, triggers proliferation, and inhibits apoptosis in pulmonary artery adventitial fibroblasts," *Antioxidants and Redox Signaling*, vol. 10, no. 10, pp. 1687–1697, 2008.

[41] C. Berry, C. A. Hamilton, M. J. Brosnan et al., "Investigation into the sources of superoxide in human blood vessels: angiotensin II increases superoxide production in human internal mammary arteries," *Circulation*, vol. 101, no. 18, pp. 2206–2212, 2000.

[42] P. J. Pagano, Y. Ito, K. Tornheim, P. M. Gallop, A. I. Tauber, and R. A. Cohen, "An NADPH oxidase superoxide-generating system in the rabbit aorta," *The American Journal of Physiology*, vol. 268, no. 6, pp. H2274–H2280, 1995.

[43] H. D. Wang, P. J. Pagano, Y. Du et al., "Superoxide anion from the adventitia of the rat thoracic aorta inactivates nitric oxide," *Circulation Research*, vol. 82, no. 7, pp. 810–818, 1998.

[44] B. Somoza, M. C. González, J. M. González, F. Abderrahim, S. M. Arribas, and M. S. Fernández-Alfonso, "Modulatory role of the adventitia on noradrenaline and angiotensin II responses: role of endothelium and AT2 receptors," *Cardiovascular Research*, vol. 65, no. 2, pp. 478–486, 2005.

[45] J. Chao, J. G. Wood, V. G. Blanco, and N. C. Gonzalez, "The systemic inflammation of alveolar hypoxia is initiated by alveolar macrophage-borne mediator(s)," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 41, no. 5, pp. 573–582, 2009.

[46] D. L. Burke, M. G. Frid, C. L. Kunrath et al., "Sustained hypoxia promotes the development of a pulmonary artery-specific chronic inflammatory microenvironment," *The American Journal of Physiology*, vol. 297, no. 2, pp. L238–L250, 2009.

[47] J. Zhang, J. M. Patel, Y. D. Li, and E. R. Block, "Proinflammatory cytokines downregulate gene expression and activity of constitutive nitric oxide synthase in porcine pulmonary artery endothelial cells," *Research Communications in Molecular Pathology and Pharmacology*, vol. 96, no. 1, pp. 71–88, 1997.

[48] S.-Y. Lam, Y. Liu, K.-M. Ng et al., "Chronic intermittent hypoxia induces local inflammation of the rat carotid body via functional upregulation of proinflammatory cytokine pathways," *Histochemistry and Cell Biology*, vol. 137, no. 3, pp. 303–317, 2012.

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Review Article

The Endothelial ADMA/NO Pathway in Hypoxia-Related Chronic Respiratory Diseases

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Since its discovery, many adhere to the view that asymmetric dimethylarginine (ADMA), as an inhibitor of the synthesis of nitric oxide (NO), contributes to the pathogenesis of various diseases. Particularly, this is evident in disease of the cardiovascular system, in which endothelial dysfunction results in an imbalance between vasoconstriction and vasodilatation. Even if increased ADMA concentrations are closely related to an endothelial dysfunction, several studies pointed to a potential beneficial effect of ADMA, mainly in the context of angioproliferative disease such as cancer and fibrosis. Antiproliferative properties of ADMA independent of NO have been identified in this context. In particular, the regulation of ADMA by its degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) is the object of many studies. DDAH is discussed as a promising therapeutic target for the indirect regulation of NO. In hypoxia-related chronic respiratory diseases, this controversy discussion of ADMA and DDAH is particularly evident and is therefore subject of this review.

1. Introduction: The Endothelial ADMA/NO Pathway

1.1. Nitric Oxide. Endothelial-derived NO is known to be the major mediator regulating vasomotor tone. NO is involved in a wide range of mechanisms with regulatory function, including inhibition of platelet adhesion and aggregation, of monocyte adhesion and of smooth muscle cell proliferation. In this way, NO plays a crucial role in vascular homeostasis. NO is produced by nitric oxide synthase (NOS) enzymes [1]. There are three distinct isoforms which catalyze the formation of NO from the substrate L-arginine and O_2 with L-citrulline being produced as a second product. The distinct isoforms differ in their tissue and cell type distribution as well as in their regulatory mechanisms [2]. The three isoforms are neuronal NOS (NOS1, nNOS) [3], inducible NOS (NOS2, iNOS) [4], and the endothelial NOS (NOS3, eNOS) [5]. Among others, nNOS is mainly expressed in the central and peripheral nervous system, kidney, pancreas, and skeletal muscle [6]. The inducible form of NOS was initially identified as a mediator of innate immunity and macrophages and could be induced in different cell types like vascular smooth muscle cells, renal tubular epithelium, hepatocytes, and mesangial cells [7]. The expression of the eNOS is largely restricted to the vascular endothelial cells and mainly in medium- and large-sized arteries and arterioles [7].

1.2. Nitric Oxide and Oxygen. Not only is the production of NO oxygen dependant but also NO plays a very important role in the regulation of O_2 delivery through vasomotor control locally and cardiovascular and respiratory response centrally. O_2 is well known for its important function in cellular energy production. O_2 carrying capacity and saturation of the blood flow are the principle determinants

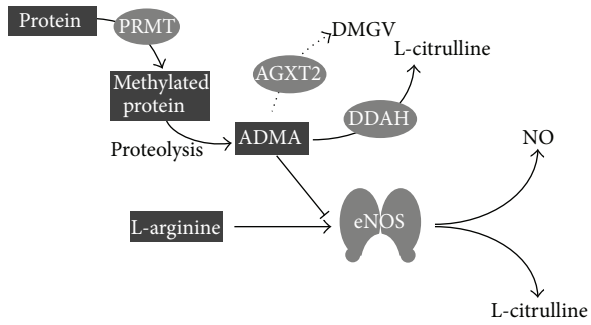

Figure 1: Endothelial L-arginine/NO pathway. L-arginine residues in proteins are methylated by protein-arginine methyltransferases (PRMT), after proteolysis ADMA is released and could replace L-arginine from the binding site at the NOS. ADMA is mainly degraded by dimethylarginine dimethylaminohydrolases (DDAH) to L-citrulline. The degradation of ADMA by alanine-glyoxylate aminotransferase 2 (AGXT-2) to α-keto-δ-(N(G),N(G)-dimethylguanidino)valeric acid (DMGV) is described as an alternative way which metabolized ADMA only to a very small proportion. This ADMA/AGXT-2 pathway is not object of this review.

of tissue O_2 delivery. Therefore, NO plays a major role in regulating vascular tone and organ function in the setting of hypoxia [8]. Paradoxically, hypoxic environment decreases eNOS expression and function which shows us that the view of NO as only a regulator of the vasotonus or blood pressure is too simple. In the last years, the NO signal cascade is discussed as a "sense-and-response" pathway for reduced O_2 bioavailability through an interaction with the O_2-sensing pathway (for review see [9]). Another example pointing to the complexity of the role of the L-arginine/NO pathway under hypoxic conditions was shown by Howell et al. [10]. They could demonstrate that supplementation of L-arginine promotes angiogenesis within the gas exchange region of hypoxic lungs and it attenuated the development of pulmonary hypertension in rats in a NO-independent manner [10]. This shows that, beyond the function as a substrate for the NOS, L-arginine seems to have additional proangiogenic properties especially in the pulmonary circulation.

1.3. Endogenous NOS Inhibitors in Cardiovascular Disease. N-guanidino-dimethylation of L-arginine residues in proteins by protein-arginine methyltransferases (PRMTs) and subsequent proteolysis lead to the release of free dimethylated L-arginine analogous in the tissue and plasma (Figure 1) [11]. ADMA is known to be an inhibitor of all three isoforms of NOS. It competes with L-arginine for the binding site in the active centre of NOS [12]. Furthermore, ADMA can "uncouple" the NOS by shifting the balance of NO generation to the side of superoxide production. *In vitro* and *in vivo* studies demonstrate that an increase in ADMA could lead to an impaired NO bioavailability as well as an increase in the formation of reactive oxygen species (ROS) [13]. Another dimethylated L-arginine analogue is the symmetric dimethylarginine (SDMA), but its role in the endothelial NO pathway is still unclear. SDMA and ADMA are able to interfere with the substrate availability of NOS by inhibiting the accordant transmembrane cationic amino acid transport (CAT) system of L-arginine, but the IC_{50} values are above the estimated endogenous ADMA and SDMA concentrations [14]. In a large number of prospective clinical studies, ADMA has been characterized as a predictor of major cardiovascular events and mortality in patients with low, medium, and high cardiovascular risk [15, 16]. Some recent studies suggest that SDMA is also associated with cardiovascular events [17, 18] and we have shown that SDMA, but not ADMA, is predictive of all-cause mortality after ischemic stroke [19, 20]. Almost 80% of ADMA is enzymatically hydrolyzed by the dimethylarginine dimethylaminohydrolase (DDAH). DDAH is expressed in two isoforms, DDAH-1 and DDAH-2, which are characterized by distinct tissue distribution, are encoded by different genes, and may exert distinct functional roles [21, 22]. Overexpression of DDAH-1 or DDAH-2 rescues mice from adverse effects of ADMA infusion and improves recovery from vascular damage [23–28]. Transient siRNA-mediated knock-down experiments in rats imply specific functions of DDAH isoforms. Based on these experiments, it appears that DDAH-1 is the dominant form regulating plasma ADMA levels, whereas DDAH-2 appears to be required for actelycholine-dependent vasodilatation [22].

The indirect regulation of the NO bioavailability by varying the ADMA concentrations is discussed as a therapeutic option in various diseases [29]. The regulation of ADMA concentration is possible on different levels. An increase of ADMA formation by enhanced PRMT activity could be seen in the context of different types of human cancer pointing to an decreased NO bioavailability [30]. An enhanced PRMT activity could be also seen in various chronic respiratory diseases leading to the discussion that protein methylation might be a mechanism with therapeutic potential [31]. The effect on NO formation due to increased ADMA concentrations by a reduced ADMA degradation could be recently demonstrated by Ghebremariam and colleagues. They identified a potent DDAH inhibitor which significantly increased intracellular ADMA levels and reduced lipopolysaccharide-induced NO production in endothelial cells [32].

However, it is undisputed that increased concentrations of ADMA and SDMA in tissue and plasma in human as well as in rodents are associated with an unfavorable course of various cardiovascular diseases to an increased mortality. The causing mechanism for ADMA is plausibly the inhibition of the NO production which results in an endothelial dysfunction, but why SDMA is associated with an unfavorable outcome is still unclear. The correlations of ADMA and SDMA with cardiovascular diseases are discussed elsewhere [33–35]. This review will focus especially on the respiratory system and the effects of hypoxia on the endothelial ADMA/NO pathway.

2. Clinical Perspective: The ADMA/NO Pathway and Endothelial Function in the Respiratory System—For Better or Worse?

2.1. Pulmonary Arterial Hypertension. It is undisputed that in the healthy lung NO plays a key role in maintaining

the ventilation/perfusion matching as a response of local hypoxia. In regions of low ventilation the NO levels are low resulting in a vasoconstriction causing the blood flow directed towards well-ventilated regions with high levels of NO to ensure efficient oxygenation of the blood. In patients with pulmonary arterial hypertension (PAH), activity of NOS is reduced compared with those of controls leading to a mismatch in the ventilation/perfusion system [36]. ADMA as a natural occurring inhibitor of NOS is increased in patients with PAH and is associated with unfavorable pulmonary hemodynamics and worse outcome in these patients [37]. The underlying mechanism is a decreased expression and activity of DDAH-2 which was shown in lungs from patients with idiopathic pulmonary arterial hypertension (IPAH) as well as in lungs of Monocrotaline-treated rats [38]. However, an increased vasoconstriction in the pulmonary circulation is only one aspect in the complex pathogenesis of PAH. PAH arises from a combination of pulmonary vasoconstriction, from vascular wall remodeling, from in situ thrombosis, and, in advanced stage of disease, from complex vascular (plexiform) lesion resembling neoangiogenesis within completely obliterated vessels [39, 40]. Besides endothelial injury, invasion of the intima by fibroblast-like cells and enhanced matrix deposition, the proliferation of endothelial cells, are responsible for the intimal changes in the vasculature resulting in hypoxemia what than contributes to the progression of the progression of PAH [41]. NO is a potent stimulator of endothelial cell proliferation, migration, and angiogenesis [42]. Inhibition of NO generation by ADMA in endothelial cells leads to enhanced apoptosis [43]. In the lung phosphodiesterase (PDE) isoenzymes—especially PDE-3 and PDE-4—are important regulators of the cAMP degradation and are upregulated in experimental models of PAH [43]. It has been shown that cAMP-elevating agents enhance EC function, especially angiogenesis [44]. Treatment of endothelial cells with a combined PDE-3/4 inhibitor significantly decreased this ADMA-induced apoptosis by regulating DDAH-2 activity in a cAMP-dependent manner [43].

Drugs targeting the NO pathway are of great interest in the therapy of PAH. Inhaled NO or NO-donors are suitable for short-term use, but due to the development of tolerance, the significant number of nonresponders, and the risk of a rebound effect, NO and NO-donors are not suitable as long-term treatment. Targeting the NO-sGC-cGMP axis downstream of NO seems to be more promising. Inhibiting the degradation of cGMP by inhibiting the PDE-5 has been approved for the treatment of PAH [45–47]. Stimulation of the NO receptor soluble guanylate cyclase (sGC) with Riociguat is another therapeutic strategy acting independently of NO levels [48]. Riociguat has shown promising results in clinical trials and might be available soon [49, 50].

2.2. Asthma. Bronchial inflammation, especially in allergic asthma, is triggered by a cascade of proinflammatory mediators including NO [51]. Besides the infiltration and activation of inflammatory cells in the airways, one key pathogenic feature in asthma is the hyperresponsiveness of the airways starting from airway endothelial and smooth muscle cells. Due to an increased iNOS expression in the lung epithelium [52], the expired NO levels are increased in asthmatic patients [53], but in contrast to that the local bioavailability of L-arginine [54] as well as the NO production by the constitutive NOS in smooth muscle cells is reduced [55]. Consistent with this an alteration in L-arginine metabolism especially in the L-arginine degradation by arginase is associated with airflow abnormalities in patients with severe asthma [56]. This mismatch in the L-arginine/NO pathway contributes to the hyperresponsiveness in the airways of asthmatic patients. Looking at the ADMA/NO pathway in these patients, it is clearly shown that ADMA is increased in peripheral compartments (e.g., plasma) as well as locally in lung specimens, sputum, and exhaled breath condensate [57]. The increase in ADMA is often accompanied by a reduced L-arginine bioavailability leading to a decreased L-arginine/ADMA ratio which is proposed to be a novel index reflecting an imbalance in NOS activity caused by an accumulation of ADMA [58]. In a mouse model of allergic asthma, increased ADMA concentrations in the lung caused by a decreased DDAH expression potentiate airway inflammation via modulation of iNOS [59, 60]. This again provides support that the ADMA/DDAH pathway seems to be the key regulator of the L-arginine/NO signaling in a diseased vasculature.

2.3. Chronic Obstructive Pulmonary Disease. Approximately 600 million people worldwide are suffering from chronic obstructive lung disease (COPD). COPD is the fourth leading cause of death mainly due to tobacco smoking and must call a global problem. COPD is the only disease whose incidence is increasing constantly. The pathophysiological concept suggests an inflammatory burden and remodeling in the lung leading to the destruction of the elastic architecture of the lung and enlargement of distal air space [61]. In about 30–70% of these patients, the COPD is accompanied by pulmonary hypertension [62]. Pulmonary hypertension is often thought to be a consequence of hypoxic conditions in combination with tobacco smoking in patients with advanced COPD. The impact of a vascular pathology for the pathogenesis is still unresolved. Oxidative and nitrosative stress have been suggested as factors involved in the chronic inflammation and enhanced proliferation processes in the pathogenesis of COPD [63]. In induced sputum samples of unstable COPD patients, an increased number of cells expressing iNOS and nitrotyrosine could be counted [63]. Nitrotyrosine is the reaction product of tyrosine residues and peroxynitrite, which is formed by the reaction of NO and superoxide [51]. In this context, NO derived from iNOS is a key player and is closely linked to the vascular pathology to emphysema development. In an established experimental COPD mouse model, the inhibition of iNOS by L-NIL as well as the genetic depletion of iNOS protected against the development of PH and Emphysema. In this context the iNOS downregulation was associated with a reduced number of proinflammatory cells like granulocytes, macrophages, activated macrophages, and T cells [64]. Interestingly, after

full establishment of emphysema, iNOS inhibition was associated with curative restored lung structure and lung function [64]. About the role of the ADMA/NO axis in this context is little known. In exhaled breath condensate of patients with COPD increased ADMA concentrations have been seen [65]. The NOS inhibitory capacity of ADMA leads to the assumption of a regulatory function of ADMA regarding the NO bioavailability in COPD. But this needs to be verified.

2.4. Idiopathic Pulmonary Fibrosis. Idiopathic pulmonary fibrosis (IPF) characterized by an injury of alveolar epithelium, alveolar inflammation, and increased proliferation of fibroblast is the most common and aggressive form of lung fibrosis. In the process, IPF could result in a progressive loss of alveolar capillaries and lung architecture, which dramatically affects sufficient oxygenation and is therefore associated with high morbidity and mortality [66]. IPF is not typically defined as a vascular disease. However, the final stage of IPF resulted in hypoxia which could then effect secondarily the vascular system. But up to now little is known about the pathomechanisms and the involvement of the vascular system especially of the endothelial system. However, what is certain is the involvement of the inducible NOS in the pathophysiology of IPF. Lung protein levels of iNOS were three times higher in patients with IPF compared with control donors and were observed close to fibrotic scares, thickened septa, and fibroblast foci. Interestingly, this iNOS expression was accompanied by an increased colocalized expression of DDAH-2 [67]. This colocalization suggests an ADMA-related regulation of the iNOS. In mice with bleomycin-induced fibrosis, an increased immunoreactivity of DDAH-1 and DDAH-2 was detected in the endothelium, inflammatory cells, fibroblasts, airway epithelial cells, and alveolar epithelial cells. This increase of DDAH was associated with decreased ADMA levels. Surprisingly DDAH inhibition by the L-291 suppressed the abnormal proliferation of alveolar epithelial cells in IPF and induced apoptosis in an ADMA-dependent manner. Additionally, DDAH inhibition as well as iNOS inhibition reduced collagen production by fibroblasts and improved lung function in bleomycin-treated mice [67]. This example of IPF demonstrated the controversy of increased ADMA concentration in the diseased respiratory vascular system.

2.5. Lung Cancer. Intensive investigation has been conducted on the role of the NO pathway in cancer. NO plays a role in cellular proliferation, migration, induction of epithelial-mesenchymal transition, angiogenesis, and apoptosis of cancer cells. Increased NO concentration can be detected in the microenvironment of many solid cancers. However, the role of NO appears ambiguous and may indicate a biphasic nature of NO-mediated cellular effects depending on its concentration at the site of cancer cells, the chemical redox environment, and the duration of NO exposure; that is, NO can act pro- and antitumorigenic (reviewed by [68]). In non-small-cell lung cancer, increased expression of iNOS has been observed in tumor tissue, and patients exhale elevated NO levels [69]. Expression of iNOS contributes to the urethane-induced and to genetically, Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation-induced lung carcinogenesis whereas inhibition of iNOS reduced carcinogenesis in animal cancer models [70, 71]. Hypoxia occurs within growing and expanding tumor tissue and may drive (neo-)angiogenesis, and cause, if not caught up by novel vessel generation, tumor necrosis due to lacking tumor cell nutrition and oxygenation. The histopathological extend of intratumoral necrosis is associated with unfavorable prognosis in lung cancer and other entities such as colorectal cancer [72, 73]. Hypoxia stimulates iNOS expression and NO production and hereby may contribute to tumor blood supply [74]. Interestingly, intravenous administration of the NOS inhibitor N^{Ω}-nitro-L-arginine (L-NNA) reduced the tumor blood supply in patients with non-small-cell lung cancer providing the early clinical evidence that inhibition of NOS has antivascular activity in cancer [75]. Preclinically, privation of blood flow caused by NOS inhibition can be restored by administration of L-arginine underlying the NO dependence of cancerous vascularity [76]. A small study showed that plasmatic levels of the intrinsic NOS inhibitor ADMA are increased in patients with small and non-small-cell lung cancer without concomitant cardiovascular diseases as compared to healthy subjects [30]. In this study comparable elevation of ADMA has been observed in other epithelial cancers such as gastric and breast cancer indicating a vascular response involving the ADMA/NO pathway in cancer patients [30]. The biological significance and prognostic role of ADMA in lung cancer are yet unknown.

2.6. Obstructive Sleep Apnea Syndrome. Obstructive sleep apnea syndrome (OSAS) is defined by the presence of symptoms such as daytime sleepiness in conjunction with a significant quantity of obstructive events occurring during sleep. The registration of ventilator event includes episodes of apneas and hypopneas and increased upper airway resistance [77]. OSAS has been found to be an independent risk factor for cardiovascular events [78]. Episodes of desaturation-reoxygenation during night are a typical pattern. This sequence, defining intermittent hypoxia, causes the generation of oxidative stress such as production of ROS which contributes to systemic inflammation found in these patients [77]. Oxidative stress and inflammatory process such as increased leukocyte adhesion via expression of adhesion molecules promote endothelial damage and dysfunction [79]. Thus, impaired endothelium-dependent vasodilatation is typically found in patients with OSAS [80] and can partly be reversed by continuous positive airway pressure (CPAP) therapy indicating a crucial pathophysiological link between the endothelial dysfunction and intermittent hypoxemia in OSAS [81]. The NO metabolism has been strongly implicated in this relationship. Levels of circulating NO measured as serum nitrites and nitrates were significantly lower in patients with OSAS and correlated negatively with parameters of disease severity in these patients [82]. Furthermore, plasma ADMA levels are elevated in patients with OSAS irrespective of the presence of further cardiovascular risk factor

[83]. Following CPAP therapy with significant reduction of intermittent episodes of hypoxemia, levels of NO and ADMA can be restored [82, 84].

3. Clinical Perspective: Hypoxia and Endothelial Function in the Respiratory System

Alveolar hypoxia redirects the capillary blood flow to areas of higher oxygen availability by hypoxia-induced vasoconstriction of pulmonary arterial vessels [85, 86]. This mechanism accounts for a sufficient maintenance of blood oxygenation. Cells of the precapillary smooth muscle layer of vessels located at the entrance of the acinus are thought to be the sensor and effector cell-type in this mechanism in a calcium-dependent manner (reviewed by [87]). However, hypoxia-induced pulmonary vasoconstriction can be abolished by denudation of the endothelial layer as demonstrated in porcine small pulmonary vessels [88]. This observation underlies the complexity of the intercellular regulatory network in response to acute hypoxia. Mediators derived from the pulmonary arterial endothelial cells critically regulate the vascular tone in response to hypoxia (reviewed by [89]). Chronic respiratory diseases such as COPD or fibrosis are associated with sustained systemic hypoxemia by altered gas-exchange due to increased diffusion distance, poor ventilation, or loss of alveolar structures. In contrast to acute or subacute hypoxia, with sustained hypoxia, a temporary vasodilatation has been described, followed by a secondary vasoconstrictor response [87]. The response of endothelial cells differs from that followed by acute hypoxia, while under acute hypoxic condition pulmonary endothelial cells have been shown to slow down their cell cycle progression (but did not arrest), and under chronic hypoxic conditions pulmonary endothelial cells exhibited enhanced proliferation [90, 91]. On cellular level, exposure to chronic hypoxia leads to progressive pulmonary vascular remodeling associated with increased vascular resistance and development of a pulmonary hypertension phenotype [92]. In humans vascular remodeling consisting of thickened pulmonary vessels and resulting in elevated pulmonary arterial resistance is evident under chronic hypoxic conditions in high altitude [93]. In patients with underlying hypoxia-related respiratory disease, such as COPD, mild elevation of pulmonary arterial pressure is frequent and associated with unfavorable prognosis regarding all-cause mortality [62]. However, in this relationship clinical data revealed no clear correlation between grade of hypoxemia and magnitude of pulmonary hypertension indicating further mechanism, in addition to hypoxemia, that leads to vascular remodeling [94]. A possible additional aspect might be the link between chronic lung inflammation, systemic inflammation, and as a result vascular inflammation which leads to dysfunctional endothelial cells and causes cardiovascular morbidity [95].

4. Hypoxia as a Mediator of ADMA/NO-Related Endothelial Dysfunction: Little Is Known

Clinical as well as experimental data clearly show that the dysregulation of the ADMA/NO pathway plays a crucial role in the development and/or progression of hypoxia associated chronic respiratory diseases. But the underlying molecular mechanisms can be varied and are not really clear. Hypoxia as a cause for vascular changes or hypoxia as a consequence of chronic respiratory diseases seems to play a major role in the regulation of the ADMA/NO pathway.

Acute and chronic changes in oxygen levels lead to the activation of comprehensive sense and response mechanisms in the whole organism or locally in different organs and tissues. The heart of these response mechanisms is the hypoxia-inducible factor (HIF) which consists of a HIF-1 α-subunit (HIF-1α) and a nuclear β-subunit (HIF-1β) [96]. Under normoxic conditions, HIF-1α is bound by Von Hippel-Lindau protein (pVHL) [97]. The binding of pVHL is dependent on the hydroxylation of a specific proline residue in HIF-1α by the oxygen-dependent prolyl hydroxylase (PHD) 2. PHD-2 uses O_2 as a substrate and thus PHD-2 activity is inhibited under hypoxic conditions [98]. In this bound and inactive form HIF-1α is proteasomal degraded by an ubiquitin ligase (Figure 2(a)) [99]. HIF-2α, a paralog of HIF-1α, is found in vertebrates and is also regulated by prolyl hydroxylation. HIF-2α also dimerizes with HIF-1β and plays an important role in erythropoiesis, vascularization, and pulmonary development [100]. The pathological consequences of HIF-1 dysregulation in chronic diseases include a wide range of both protective and pathogenic responses. Diseases in which HIF-1 mediates protective responses are coronary artery disease [101], peripheral arterial disease [102], Colitis [103], and organ transplant rejection [104]. In cancer [105] and chronic respiratory diseases like PAH [106] and OSAS [107] HIF-1 activity contributes to the pathogenesis of the disease.

Interestingly, there seems to be a mutual regulation of NO signaling and hypoxic HIF signaling. One key mechanism by which NO regulates cellular targets or hypoxia signaling is S-nitrosylation [108, 109]. Components of the HIF-1α signaling are targets for S-nitrosylation resulting in a stabilization of HIF-1α under normoxic conditions [109, 110]. Direct S-nitrosylation of the Cys-533 of HIF-1α prevents the binding of pVHL and the following polyubiquitination (Figure 2(b)). S-nitrosylation of Cys-162 of pVHL prevents the binding of pVHL to HIF-1α (Figure 2(b)) and also inhibits its ability to mediate the polyubiquitination of HIF-1α [110]. Additionally, it has been shown that under hypoxic conditions different concentrations of NO—as an inhibitor of the mitochondrial cytochrome c oxidase—have different effects on HIF-α stabilization. Concentrations of NO < 400 nM resulted in a decrease of HIF-α stabilization whereas NO concentrations of $> 1\,\mu$M caused a stabilization of HIF-α [111]. This could be explained by an increase in oxygen-independent PHD-dependent degradation of HIF-α [112].

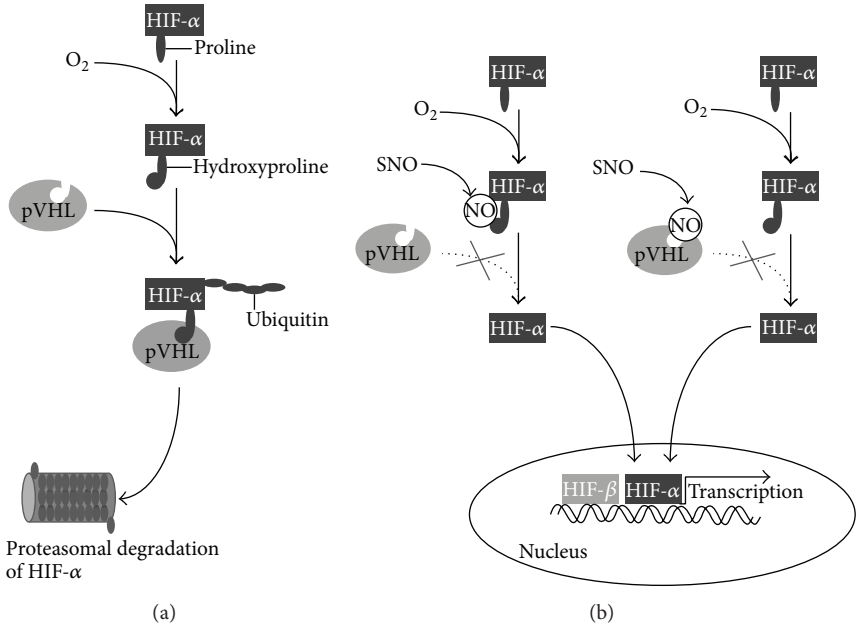

Figure 2: S-nitrosylation of HIF-α and pVHL. (a) In the presence of oxygen proline, residues of HIF-α are hydroxylated. This leads to the polyubiquitination of the pVHL-HIF-α complex resulting in the proteasomal degradation of HIF-α. (b) S-nitrosylation of HIF-α prevents the binding of pVHL and thereby the polyubiquitination of HIF-α. S-nitrosylation of pVHL also inhibits the ability to mediate the polyubiquitination of HIF-α. In both cases HIF-α is not degraded but translocated into the nucleus, dimerizes with the HIF-β subunit, and induces the transcriptional activation of target genes.

Additionally NO can also inhibit PHD activity. It competes with O_2 for Fe^{2+} at the catalytic domain of PHDs and supports the stability of HF-1α [113]. The molecular mechanisms by which hypoxia could regulate NO production in the endothelium are diverse. They range from transcriptional and epigenetic modifications to posttranscriptional and posttranslational modifications of the NOS (reviewed by [9]). This direct interaction between NO signaling and hypoxia signaling is one regulatory possibility. In pulmonary endothelial cells hypoxia can also inhibit the substrate availability of the eNOS by inhibiting the transport of L-arginine into the endothelial cell [114]. Additionally, we and others proposed that DDAH is the key determinant of intracellular ADMA concentrations and that the regulation of DDAH could therefore modulate NO bioavailability indirectly [29, 32, 115]. In 2001 Murray-Rust et al. [116] identified a Cys-His-Glu catalytic triad and Leiper et al. showed in 2002 that the presence of the reactive cysteine residue is directly regulated by NO mediated reversible S-nitrosylation [117]. So, under circumstances when NO generation increases, NO mediated S-nitrosylation inhibited DDAH activity, which leads to accumulation of ADMA and inhibition of NOS. It is conceivable that this cycle is active in all normoxic states of a modified NO generation as well as in the status of hypoxia. It recently has been shown that DDAH-1 overexpression in mice decreased sustained hypoxia-induced pulmonary vasoconstriction but did not alter the vascular response to acute or chronic hypoxia. This effect of DDAH-1 overexpression could be partly explained by an ADMA-induced inhibition of the NO pathway [118]. This study in combination with the clinical data on DDAH in chronic respiratory diseases supports the fact that hypoxia mediate DDAH activity, but the molecular mechanisms behind this hypoxia-mediated regulation are still lacking. It is generally accepted that hypoxia is associated with a high burden of ROS. One possible link between hypoxia and DDAH regulation might be the hypoxia-induced formation of ROS. Ito et al. showed that DDAH activity is inhibited by oxLDL in endothelial cells [119] leading to increased ADMA concentrations. In addition, the incubation of cultured endothelial cells with glucose resulted in an impaired DDAH activity and subsequently increased ADMA levels. This glucose-mediated effect was inhibited by antioxidants [120]. The impact of oxidative stress and DDAH is reviewed in detail by Sydow and Münzel 2003 [121]. The regulation of DDAH by ROS occurs mainly at the level of enzyme activity. There are some studies describing the genetic regulatory promoter regions of DDAH-1 and DDAH-2 and the mediated effects through activation of these regions under normoxic conditions. Both promoter regions of DDAH-1 and DDAH-2 contain a sterol response element which could bind the statin-induced transcription factor sterol response element binding protein (SREBP). Ivashchenko et al. demonstrated a reciprocal regulation by SREBP-2 and SREBP-1 of DDAH-1 and could therefore explain the positive ADMA-lowering effect by simvastatin which might therefore contribute to the vasculoprotective effect of statins [122]. Hasegawa et al. could identify a specific protein 1 (SP1) binding site in the DDAH-2 promoter which is responsible for the DDAH-induced expression and secretion of the vascular endothelial growth factor (VEGF), resulting

in an increase in proliferation and migration of endothelial cells. This effect is not dependent on the ADMA/NO pathway [123]. Jung et al. showed that Vaspin, an adipocitokine expressed in the visceral adipose tissue, mediated its antiatherogenic effect by a STAT-3 activation of the DDAH-2 promoter resulting in a decreased ADMA-induced inhibition of eNOS [124]. Another promoter modification of DDAH-2 is described by Eikelboom et al. They showed that Hyperhomocysteinemia, which is also associated with an increased risk for cardiovascular diseases [125], leads to a dose-dependent hypermethylation of the CpG island in the DDAH-2 promoter region. This hypermethylation was associated with an impaired mRNA expression of DDAH-2 [126]. These studies lead to the suggestion that there might be also a genetic regulation of the ADMA/DDAH pathway via hypoxia but the molecular crosslink between DDAH and hypoxia signaling besides ROS is not known so far.

5. The ADMA/NO Pathway: Therapeutic Potential in Respiratory Diseases?

The direct, therapeutic regulation of NOS in the field of cardiovascular diseases has been long discussed as a novel therapeutic strategy. Especially in chronic respiratory lung diseases, both beneficial and deleterious effects of NO have been shown in the airways. But up to now there is no potential direct NOS regulator for the treatment of cardiovascular diseases including chronic respiratory diseases available. The therapeutic regulation of ADMA via DDAH might be another possible mechanism to regulate NOS and therefore NO bioavailability indirectly. However, the therapeutic potential of DDAH is controversial. From many clinical studies we know that increased ADMA concentrations promote an endothelial dysfunction and remodeling processes in the lung. There is evidence that ADMA contributes to the pathogenesis of various diseases and that the inhibition of its degradation has protective properties. The positive impact of DDAH inhibition and therefore increased ADMA concentration has been already discussed in the setting of idiopathic pulmonary fibrosis [32, 127] and Endotoxic shock [128]. It is also conceivable that ADMA is anyhow involved in the local hypoxia-induced pulmonary vasoconstriction resulting in the maintenance of the ventilation/perfusion match in the lung. However, the clinical relevance of a regulation of the endothelial ADMA/NO pathway needs to be evaluated and the development of DDAH regulators is a promising approach as new therapeutic targets in some chronic respiratory diseases.

Abbreviations

ADMA: Asymmetric dimethylarginine
AGXT: Alanine-glyoxylate aminotransferase
CAT: Cationic amino acid transporter
cAMP: Cyclic adenosine monophosphate
cGMP: Cyclic guanosine monophosphate
COPD: Chronic obstructive pulmonary disease
CPAP: Continuous positive airway pressure
DDAH: Dimethylarginine dimethylaminohydrolase
eNOS: Endothelial nitric oxide synthase
HIF: Hypoxia-induced factor
iNOS: Inducible nitric oxide synthase
IPAH: Idiopathic pulmonary arterial hypertension
IPF: Idiopathic pulmonary fibrosis
KRAS: Kirsten rat sarcoma viral oncogene homolog
L-NNA: N-nitro-L-arginine
nNOS: Neuronal nitric oxide synthase
NO: Nitric oxide
NOS: Nitric oxide synthase
OSAS: Obstructive sleep apnea syndrome
PAH: Pulmonary arterial hypertension
PDE: Phosphodiesterase
PHD: Prolyl hydroxylase
ROS: Reactive oxygen species
SDMA: Symmetric dimethylarginine
sGC: Soluble guanylate cyclase
SREBP: Statin-induced transcription factor sterol response element binding protein
SP1: Specific protein 1
STAT: Signal transducer and activator of transcription 3 (acute-phase response factor)
VEGF: Vascular endothelial growth factor
pVHL: Protein von Hippel-Lindau.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] S. Moncada and A. Higgs, "The L-arginine-nitric oxide pathway," *The New England Journal of Medicine*, vol. 329, no. 27, pp. 2002–2012, 1993.

[2] T. Michel and O. Feron, "Nitric oxide synthases: which, where, how, and why?" *The Journal of Clinical Investigation*, vol. 100, no. 9, pp. 2146–2152, 1997.

[3] D. S. Bredt, C. E. Glatt, P. M. Hwang, M. Fotuhi, T. M. Dawson, and S. H. Snyder, "Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase," *Neuron*, vol. 7, no. 4, pp. 615–624, 1991.

[4] C. J. Lowenstein, C. S. Glatt, D. S. Bredt, and S. H. Snyder, "Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 15, pp. 6711–6715, 1992.

[5] K. Sase and T. Michel, "Expression and regulation of endothelial nitric oxide synthase," *Trends in Cardiovascular Medicine*, vol. 7, no. 1, pp. 28–37, 1997.

[6] J. N. Wilcox, R. R. Subramanian, C. L. Sundell et al., "Expression of multiple isoforms of nitric oxide synthase in normal and

atherosclerotic vessels," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 17, no. 11, pp. 2479–2488, 1997.

[7] S. Moncada, R. M. J. Palmer, and E. A. Higgs, "Nitric oxide: physiology, pathophysiology, and pharmacology," *Pharmacological Reviews*, vol. 43, no. 2, pp. 109–142, 1991.

[8] G. L. Semenza, "Mechanisms of disease: oxygen sensing, homeostasis, and disease," *The New England Journal of Medicine*, vol. 365, no. 6, pp. 537–547, 2011.

[9] J. J. Ho, H. S. Man, and P. A. Marsden, "Nitric oxide signaling in hypoxia," *Journal of Molecular Medicine*, vol. 90, pp. 217–231, 2012.

[10] K. Howell, C. M. Costello, M. Sands, I. Dooley, and P. McLoughlin, "L-Arginine promotes angiogenesis in the chronically hypoxic lung: a novel mechanism ameliorating pulmonary hypertension," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 296, no. 6, pp. L1042–L1050, 2009.

[11] C. T. L. Tran, J. M. Leiper, and P. Vallance, "The DDAH/ADMA/NOS pathway," *Atherosclerosis Supplements*, vol. 4, no. 4, pp. 33–40, 2003.

[12] J. Leiper and P. Vallance, "Biological significance of endogenous methylarginines that inhibit nitric oxide synthases," *Cardiovascular Research*, vol. 43, no. 3, pp. 542–548, 1999.

[13] L. J. Druhan, S. P. Forbes, A. J. Pope, C.-A. Chen, J. L. Zweier, and A. J. Cardounel, "Regulation of eNOS-derived superoxide by endogenous methylarginines," *Biochemistry*, vol. 47, no. 27, pp. 7256–7263, 2008.

[14] J. Strobel, M. Mieth, B. Endreß et al., "Interaction of the cardiovascular risk marker asymmetric dimethylarginine (ADMA) with the human cationic amino acid transporter 1 (CAT1)," *Journal of Molecular and Cellular Cardiology*, vol. 53, pp. 392–400, 2012.

[15] R. H. Böger, H. G. Endres, E. Schwedhelm et al., "Asymmetric dimethylarginine as an independent risk marker for mortality in ambulatory patients with peripheral arterial disease," *Journal of Internal Medicine*, vol. 269, no. 3, pp. 349–361, 2011.

[16] R. H. Böger, R. Maas, F. Schulze, and E. Schwedhelm, "Asymmetric dimethylarginine (ADMA) as a prospective marker of cardiovascular disease and mortality-An update on patient populations with a wide range of cardiovascular risk," *Pharmacological Research*, vol. 60, no. 6, pp. 481–487, 2009.

[17] M. O. Gore, N. Lüneburg, E. Schwedhelm et al., "Symmetrical dimethylarginine predicts mortality in the general population: observations from the dallas heart study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, pp. 2682–2688, 2013.

[18] S. Kiechl, T. Lee, P. Santer et al., "Asymmetric and symmetric dimethylarginines are of similar predictive value for cardiovascular risk in the general population," *Atherosclerosis*, vol. 205, no. 1, pp. 261–265, 2009.

[19] N. Lüneburg, R.-A. von Holten, R. F. Töpper, E. Schwedhelm, R. Maas, and R. H. Böger, "Symmetric dimethylarginine is a marker of detrimental outcome in the acute phase after ischaemic stroke: role of renal function," *Clinical Science*, vol. 122, no. 3, pp. 105–111, 2012.

[20] F. Schulze, A. M. Carter, E. Schwedhelm et al., "Symmetric dimethylarginine predicts all-cause mortality following ischemic stroke," *Atherosclerosis*, vol. 208, no. 2, pp. 518–523, 2010.

[21] X. Hu, X. Xu, G. Zhu et al., "Vascular endothelial-specific dimethylarginine dimethylaminohydrolase-1- deficient mice reveal that vascular endothelium plays an important role in removing asymmetric dimethylarginine," *Circulation*, vol. 120, no. 22, pp. 2222–2229, 2009.

[22] D. Wang, P. S. Gill, T. Chabrashvili et al., "Isoform-specific regulation by NG,NG-dimethylarginine dimethylaminohydrolase of rat serum asymmetric dimethylarginine and vascular endothelium-derived relaxing factor/NO," *Circulation Research*, vol. 101, no. 6, pp. 627–635, 2007.

[23] H. Dayoub, V. Achan, S. Adimoolam et al., "Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence," *Circulation*, vol. 108, no. 24, pp. 3042–3047, 2003.

[24] H. Dayoub, R. Rodionov, J. P. Cooke, S. R. Lentz, and F. M. Faraci, "Human DDAH-I transgenic mice exhibit increased basal vascular NO and are protected against ADMA-induced endothelial dysfunction," *The Faseb Journal*, vol. 19, pp. A1238–A1238, 2005.

[25] K. Hasegawa, S. Wakino, S. Tatematsu et al., "Role of asymmetric dimethylarginine in vascular injury in transgenic mice overexpressing dimethylarginie dimethylaminohydrolase 2," *Circulation Research*, vol. 101, no. 2, pp. e2–e10, 2007.

[26] J. Jacobi, K. Sydow, G. von Degenfeld et al., "Overexpression of dimethylarginine dimethylaminohydrolase reduces tissue asymmetric dimethylarginine levels and enhances angiogenesis," *Circulation*, vol. 111, no. 11, pp. 1431–1438, 2005.

[27] H. Konishi, K. Sydow, and J. P. Cooke, "Dimethylarginine dimethylaminohydrolase promotes endothelial repair after vascular injury," *Journal of the American College of Cardiology*, vol. 49, no. 10, pp. 1099–1105, 2007.

[28] M. Tanaka, K. Sydow, F. Gunawan et al., "Dimethylarginine dimethylaminohydrolase overexpression suppresses graft coronary artery disease," *Circulation*, vol. 112, no. 11, pp. 1549–1556, 2005.

[29] J. Bełtowski and A. Kedra, "Asymmetric dimethylarginine (ADMA) as a target for pharmacotherapy," *Pharmacological Reports*, vol. 58, no. 2, pp. 159–178, 2006.

[30] M. Yoshimatsu, G. Toyokawa, S. Hayami et al., "Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers," *International Journal of Cancer*, vol. 128, no. 3, pp. 562–573, 2011.

[31] D. Zakrzewicz and O. Eickelberg, "From arginine methylation to ADMA: a novel mechanism with therapeutic potential in chronic lung diseases," *BMC Pulmonary Medicine*, vol. 9, article 5, 2009.

[32] Y. T. Ghebremariam, D. A. Erlanson, and J. P. Cooke, "A novel and potent inhibitor of dimethylarginine dimethylaminohydrolase: a modulator of cardiovascular nitric oxide," *Journal of Pharmacology and Experimental Therapeutics*, vol. 348, pp. 69–76, 2014.

[33] M. Anderssohn, E. Schwedhelm, N. Lüneburg, R. S. Vasan, and R. H. Böger, "Asymmetric dimethylarginine as a mediator of vascular dysfunction and a marker of cardiovascular disease and mortality: an intriguing interaction with diabetes mellitus," *Diabetes and Vascular Disease Research*, vol. 7, no. 2, pp. 105–118, 2010.

[34] R. H. Böger, A. Diemert, E. Schwedhelm, N. Lüneburg, R. Maas, and K. Hecher, "The role of nitric oxide synthase inhibition by asymmetric dimethylarginine in the pathophysiology of preeclampsia," *Gynecologic and Obstetric Investigation*, vol. 69, no. 1, pp. 1–13, 2010.

[35] E. Schwedhelm and R. H. Böger, "The role of asymmetric and symmetric dimethylarginines in renal disease," *Nature Reviews Nephrology*, vol. 7, no. 5, pp. 275–285, 2011.

[36] A. Giaid and D. Saleh, "Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension," *The New England Journal of Medicine*, vol. 333, no. 4, pp. 214–221, 1995.

[37] J. T. Kielstein, S. M. Bode-Böger, G. Hesse et al., "Asymmetrical dimethylarginine in idiopathic pulmonary arterial hypertension," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 7, pp. 1414–1418, 2005.

[38] S. Pullamsetti, L. Kiss, H. A. Ghofrani et al., "Increased levels and reduced catabolism of asymmetric and symmetric dimethylarginines in pulmonary hypertension," *The FASEB Journal*, vol. 19, no. 9, pp. 1175–1177, 2005.

[39] S. Rich and B. H. Brundage, "Pulmonary hypertension: a cellular basis for understanding the pathophysiology and treatment," *Journal of the American College of Cardiology*, vol. 14, no. 3, pp. 545–550, 1989.

[40] M. Rabinovitch, "Molecular pathogenesis of pulmonary arterial hypertension," *The Journal of Clinical Investigation*, vol. 122, pp. 4306–4313, 2012.

[41] R. M. Tuder, B. Groves, D. B. Badesch, and N. F. Voelkel, "Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension," *The American Journal of Pathology*, vol. 144, no. 2, pp. 275–285, 1994.

[42] J. P. Cooke, "A novel mechanism for pulmonary arterial hypertension?" *Circulation*, vol. 108, no. 12, pp. 1420–1421, 2003.

[43] S. S. Pullamsetti, R. Savai, M. B. Schaefer et al., "CAMP phosphodiesterase inhibitors increases nitric oxide production by modulating dimethylarginine dimethylaminohydrolases," *Circulation*, vol. 123, no. 11, pp. 1194–1204, 2011.

[44] S. Namkoong, C.-K. Kim, Y.-L. Cho et al., "Forskolin increases angiogenesis through the coordinated cross-talk of PKA-dependent VEGF expression and Epac-mediated PI3K/Akt/eNOS signaling," *Cellular Signalling*, vol. 21, no. 6, pp. 906–915, 2009.

[45] N. Galiè, H. A. Ghofrani, A. Torbicki et al., "Sildenafil citrate therapy for pulmonary arterial hypertension," *The New England Journal of Medicine*, vol. 353, no. 20, pp. 2148–2157, 2005.

[46] H. A. Ghofrani, R. Wiedemann, F. Rose et al., "Sildenafil for treatment of lung fibrosis and pulmonary hypertension: a randomised controlled trial," *The Lancet*, vol. 360, no. 9337, pp. 895–900, 2002.

[47] M. R. Wilkins, J. Wharton, F. Grimminger, and H. A. Ghofrani, "Phosphodiesterase inhibitors for the treatment of pulmonary hypertension," *European Respiratory Journal*, vol. 32, no. 1, pp. 198–209, 2008.

[48] H. A. Ghofrani and F. Grimminger, "Soluble guanylate cyclase stimulation: an emerging option in pulmonary hypertension therapy," *European Respiratory Review*, vol. 18, no. 111, pp. 35–41, 2009.

[49] H. A. Ghofrani, M. M. Hoeper, M. Halank et al., "Riociguat for chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension: a phase II study," *European Respiratory Journal*, vol. 36, no. 4, pp. 792–799, 2010.

[50] H. A. Ghofrani, N. Galie, F. Grimminger et al., "Riociguat for the treatment of pulmonary arterial hypertension," *The New England Journal of Medicine*, vol. 369, pp. 330–340, 2013.

[51] F. L. M. Ricciardolo, P. J. Sterk, B. Gaston, and G. Folkerts, "Nitric oxide in health and disease of the respiratory system," *Physiological Reviews*, vol. 84, no. 3, pp. 731–765, 2004.

[52] R. A. Robbins, P. J. Barnes, D. R. Springall et al., "Expression of inducible nitric oxide in human lung epithelial cells," *Biochemical and Biophysical Research Communications*, vol. 203, no. 1, pp. 209–218, 1994.

[53] C. Lane, D. Knight, S. Burgess et al., "Epithelial inducible nitric oxide synthase activity is the major determinant of nitric oxide concentration in exhaled breath," *Thorax*, vol. 59, no. 9, pp. 757–760, 2004.

[54] H. Maarsingh, B. E. Bossenga, I. S. T. Bos, H. H. Volders, J. Zaagsma, and H. Meurs, "L-Arginine deficiency causes airway hyperresponsiveness after the late asthmatic reaction," *European Respiratory Journal*, vol. 34, no. 1, pp. 191–199, 2009.

[55] C. R. Morris, M. Poljakovic, L. Lavrisha, L. Machado, F. A. Kuypers, and S. M. Morris Jr., "Decreased arginine bioavailability and increased serum arginase activity in asthma," *The American Journal of Respiratory and Critical Care Medicine*, vol. 170, no. 2, pp. 148–153, 2004.

[56] A. Lara, S. B. Khatri, Z. Wang et al., "Alterations of the arginine metabolome in asthma," *The American Journal of Respiratory and Critical Care Medicine*, vol. 178, no. 7, pp. 673–681, 2008.

[57] J. A. Scott, M. L. North, M. Rafii et al., "Asymmetric dimethylarginine is increased in asthma," *The American Journal of Respiratory and Critical Care Medicine*, vol. 184, no. 7, pp. 779–785, 2011.

[58] S. M. Bode-Böger, F. Scalera, and L. J. Ignarro, "The l-arginine paradox: importance of the l-arginine/asymmetrical dimethylarginine ratio," *Pharmacology and Therapeutics*, vol. 114, no. 3, pp. 295–306, 2007.

[59] E. Klein, J. Weigel, M. C. Buford, A. Holian, and S. M. Wells, "Asymmetric dimethylarginine potentiates lung inflammation in a mouse model of allergic asthma," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 299, no. 6, pp. L816–L825, 2010.

[60] T. Ahmad, U. Mabalirajan, B. Ghosh, and A. Agrawal, "Altered asymmetric dimethyl arginine metabolism in allergically inflamed mouse lungs," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 42, no. 1, pp. 3–8, 2010.

[61] P. N. Black, P. S. T. Ching, B. Beaumont, S. Ranasinghe, G. Taylor, and M. J. Merrilees, "Changes in elastic fibres in the small airways and alveoli in COPD," *European Respiratory Journal*, vol. 31, no. 5, pp. 998–1004, 2008.

[62] O. A. Minai, A. Chaouat, and S. Adnot, "Pulmonary hypertension in COPD: epidemiology, significance, and management: pulmonary vascular disease: the global perspective," *CHEST Journal*, vol. 137, no. 6, pp. 39S–51S, 2010.

[63] M. Tsoumakidou, N. Tzanakis, G. Chrysofakis, and N. M. Siafakas, "Nitrosative stress, heme oxygenase-1 expression and airway inflammation during severe exacerbations of COPD," *CHEST Journal*, vol. 127, no. 6, pp. 1911–1918, 2005.

[64] M. Seimetz, N. Parajuli, A. Pichl et al., "Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice," *Cell*, vol. 147, no. 2, pp. 293–305, 2011.

[65] I. M. di Gangi, P. Pirillo, S. Carraro et al., "Online trapping and enrichment ultra performance liquid chromatography-tandem mass spectrometry method for sensitive measurement of, "arginine-asymmetric dimethylarginine cycle" biomarkers in human exhaled breath condensate," *Analytica Chimica Acta*, vol. 754, pp. 67–74, 2012.

[66] T. E. King Jr., A. Pardo, and M. Selman, "Idiopathic pulmonary fibrosis," *The Lancet*, vol. 378, no. 9807, pp. 1949–1961, 2011.

[67] S. S. Pullamsetti, R. Savai, R. Dumitrascu et al., "The role of dimethylarginine dimethylaminohydrolase in idiopathic pulmonary fibrosis," *Science Translational Medicine*, vol. 3, no. 87, Article ID 87ra53, 2011.

[68] A. J. Burke, F. J. Sullivan, F. J. Giles, and S. A. Glynn, "The yin and yang of nitric oxide in cancer progression," *Carcinogenesis*, vol. 34, pp. 503–512, 2013.

[69] C.-Y. Liu, C.-H. Wang, T.-C. Chen, H.-C. Lin, C.-T. Yu, and H.-P. Kuo, "Increased level of exhaled nitric oxide and up-regulation of inducible nitric oxide synthase in patients with primary lung cancer," *British Journal of Cancer*, vol. 78, no. 4, pp. 534–541, 1998.

[70] L. R. Kisley, B. S. Barrett, A. K. Bauer et al., "Genetic ablation of inducible nitric oxide synthase decreases mouse lung tumorigenesis," *Cancer Research*, vol. 62, no. 23, pp. 6850–6856, 2002.

[71] H. Okayama, M. Saito, N. Oue et al., "NOS2 enhances KRAS-induced lung carcinogenesis, inflammation and microRNA-21 expression," *International Journal of Cancer*, vol. 132, pp. 9–18, 2013.

[72] D. E. B. Swinson, J. L. Jones, D. Richardson, G. Cox, J. G. Edwards, and K. J. O'Byrne, "Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables," *Lung Cancer*, vol. 37, no. 3, pp. 235–240, 2002.

[73] M. J. Pollheimer, P. Kornprat, R. A. Lindtner et al., "Tumor necrosis is a new promising prognostic factor in colorectal cancer," *Human Pathology*, vol. 41, no. 12, pp. 1749–1757, 2010.

[74] D. Fukumura, S. Kashiwagi, and R. K. Jain, "The role of nitric oxide in tumour progression," *Nature Reviews Cancer*, vol. 6, no. 7, pp. 521–534, 2006.

[75] Q.-S. Ng, V. Goh, J. Milner et al., "Effect of nitric-oxide synthesis on tumour blood volume and vascular activity: a phase I study," *The Lancet Oncology*, vol. 8, no. 2, pp. 111–118, 2007.

[76] G. M. Tozer, V. E. Prise, and D. J. Chaplin, "Inhibition of nitric oxide synthase induces a selective reduction in tumor blood flow that is reversible with L-arginine," *Cancer Research*, vol. 57, no. 5, pp. 948–955, 1997.

[77] P. Lévy, J.-L. Pépin, C. Arnaud et al., "Intermittent hypoxia and sleep-disordered breathing: current concepts and perspectives," *European Respiratory Journal*, vol. 32, no. 4, pp. 1082–1095, 2008.

[78] D. Gozal and L. Kheirandish-Gozal, "Cardiovascular morbidity in obstructive sleep apnea: oxidative stress, inflammation, and much more," *The American Journal of Respiratory and Critical Care Medicine*, vol. 177, no. 4, pp. 369–375, 2008.

[79] L. Dyugovskaya, P. Lavie, and L. Lavie, "Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients," *The American Journal of Respiratory and Critical Care Medicine*, vol. 165, no. 7, pp. 934–939, 2002.

[80] F. J. Nieto, D. M. Herrington, S. Redline, E. J. Benjamin, and J. A. Robbins, "Sleep apnea and markers of vascular endothelial function in a large community sample of older adults," *The American Journal of Respiratory and Critical Care Medicine*, vol. 169, no. 3, pp. 354–360, 2004.

[81] V. A. Imadojemu, K. Gleeson, S. A. Quraishi, A. R. Kunselman, L. I. Sinoway, and U. A. Leuenberger, "Impaired vasodilator responses in obstructive sleep apnea are improved with continuous positive airway pressure therapy," *The American Journal of Respiratory and Critical Care Medicine*, vol. 165, no. 7, pp. 950–953, 2002.

[82] M. S. M. Ip, B. Lam, L.-Y. Chan et al., "Circulating nitric oxide is suppressed in obstructive sleep apnea and is reversed by nasal continuous positive airway pressure," *The American Journal of Respiratory and Critical Care Medicine*, vol. 162, no. 6, pp. 2166–2171, 2000.

[83] A. Barceló, M. de la Peña, O. Ayllón et al., "Increased plasma levels of asymmetric dimethylarginine and soluble CD40 ligand in patients with sleep apnea," *Respiration*, vol. 77, pp. 85–90, 2009.

[84] Y. Ohike, K. Kozaki, K. Iijima et al., "Amelioration of vascular endothelial dysfunction in obstructive sleep apnea syndrome by nasal continuous positive airway pressure: possible involvement of nitric oxide and asymmetric NG, NG-dimethylarginine," *Circulation Journal*, vol. 69, no. 2, pp. 221–226, 2005.

[85] C. H. Orchard, R. Sanchea De Leon, and M. K. Sykes, "The relationship between hypoxic pulmonary vasoconstriction and arterial oxygen tension in the intact dog," *The Journal of Physiology*, vol. 338, pp. 61–74, 1983.

[86] H. L. Motley, A. Cournand, L. Werko, A. Himmelstein, and D. Dresdale, "The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man," *The American Journal of Physiology*, vol. 150, pp. 315–320, 1947.

[87] N. Sommer, A. Dietrich, R. T. Schermuly et al., "Regulation of hypoxic pulmonary vasoconstriction: basic mechanisms," *European Respiratory Journal*, vol. 32, no. 6, pp. 1639–1651, 2008.

[88] Q. Liu, J. S. K. Sham, L. A. Shimoda, and J. T. Sylvester, "Hypoxic constriction of porcine distal pulmonary arteries: endothelium and endothelin dependence," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 280, no. 5, pp. L856–L865, 2001.

[89] P. I. Aaronson, T. P. Robertson, and J. P. T. Ward, "Endothelium-derived mediators and hypoxic pulmonary vasoconstriction," *Respiratory Physiology & Neurobiology*, vol. 132, no. 1, pp. 107–120, 2002.

[90] O. Pak, A. Aldashev, D. Welsh, and A. Peacock, "The effects of hypoxia on-the cells of the pulmonary vasculature," *European Respiratory Journal*, vol. 30, no. 2, pp. 364–372, 2007.

[91] M. Tucci, S. I. Hammerman, S. Furfaro, J. J. Saukonnen, T. J. Conca, and H. W. Farber, "Distinct effect of hypoxia on endothelial cell proliferation and cycling," *The American Journal of Physiology—Cell Physiology*, vol. 272, no. 5, pp. C1700–C1708, 1997.

[92] K. R. Stenmark, K. A. Fagan, and M. G. Frid, "Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms," *Circulation Research*, vol. 99, no. 7, pp. 675–691, 2006.

[93] D. Penaloza and J. Arias-Stella, "The heart and pulmonary circulation at high altitudes: healthy highlanders and chronic mountain sickness," *Circulation*, vol. 115, no. 9, pp. 1132–1146, 2007.

[94] A. Chaouat, A.-S. Bugnet, N. Kadaoui et al., "Severe pulmonary hypertension and chronic obstructive pulmonary disease," *The American Journal of Respiratory and Critical Care Medicine*, vol. 172, no. 2, pp. 189–194, 2005.

[95] S. van Eeden, J. Leipsic, S. F. Paul Man, and D. D. Sin, "The relationship between lung inflammation and cardiovascular disease," *The American Journal of Respiratory and Critical Care Medicine*, vol. 186, pp. 11–16, 2012.

[96] G. L. Semenza, "Hypoxia-inducible factors in physiology and medicine," *Cell*, vol. 148, no. 3, pp. 399–408, 2012.

[97] M. Ivan, K. Kondo, H. Yang et al., "HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing," *Science*, vol. 292, no. 5516, pp. 464–468, 2001.

[98] A. C. R. Epstein, J. M. Gleadle, L. A. McNeill et al., "C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation," *Cell*, vol. 107, no. 1, pp. 43–54, 2001.

[99] W. G. Kaelin Jr. and P. J. Ratcliffe, "Oxygen sensing by metazoans: the central role of the hif hydroxylase pathway," *Molecular Cell*, vol. 30, no. 4, pp. 393–402, 2008.

[100] V. H. Haase, "Regulation of erythropoiesis by hypoxia-inducible factors," *Blood Reviews*, vol. 27, pp. 41–53, 2013.

[101] L. Gao, Q. Chen, X. Zhou, and L. Fan, "The role of hypoxia-inducible factor 1 in atherosclerosis," *Journal of Clinical Pathology*, vol. 65, pp. 872–876, 2012.

[102] A. Germani, C. di Campli, G. Pompilio, P. Biglioli, and M. C. Capogrossi, "Regenerative therapy in peripheral artery disease," *Cardiovascular Therapeutics*, vol. 27, no. 4, pp. 289–304, 2009.

[103] A. Robinson, S. Keely, J. Karhausen, M. E. Gerich, G. T. Furuta, and S. P. Colgan, "Mucosal protection by hypoxia-inducible factor prolyl hydroxylase inhibition," *Gastroenterology*, vol. 134, no. 1, pp. 145–155, 2008.

[104] D. S. Wilkes, "Chronic lung allograft rejection and airway microvasculature: is HIF-1 the missing link?" *The Journal of Clinical Investigation*, vol. 121, no. 6, pp. 2155–2157, 2011.

[105] Y.-P. Tsai and K.-J. Wu, "Hypoxia-regulated target genes implicated in tumor metastasis," *Journal of Biomedical Science*, vol. 19, article 102, 2012.

[106] J. Bond, D. P. Gale, T. Connor et al., "Dysregulation of the HIF pathway due to VHL mutation causing severe erythrocytosis and pulmonary arterial hypertension," *Blood*, vol. 117, no. 13, pp. 3699–3701, 2011.

[107] J. F. Garvey, C. T. Taylor, and W. T. McNicholas, "Cardiovascular disease in obstructive sleep apnoea syndrome: the role of intermittent hypoxia and inflammation," *European Respiratory Journal*, vol. 33, no. 5, pp. 1195–1205, 2009.

[108] M. W. Foster, T. J. McMahon, and J. S. Stamler, "S-nitrosylation in health and disease," *Trends in Molecular Medicine*, vol. 9, no. 4, pp. 160–168, 2003.

[109] L. A. Palmer, A. Doctor, P. Chhabra et al., "S-Nitrosothiols signal hypoxia-mimetic vascular pathology," *The Journal of Clinical Investigation*, vol. 117, no. 9, pp. 2592–2601, 2007.

[110] F. Li, P. Sonveaux, Z. N. Rabbani et al., "Regulation of HIF-1α stability through S-nitrosylation," *Molecular Cell*, vol. 26, no. 1, pp. 63–74, 2007.

[111] J. Mateo, M. García-Lecea, S. Cadenas, C. Hernández, and S. Moncada, "Regulation of hypoxia-inducible factor-1α by nitric oxide through mitochondria-dependent and -independent pathways," *Biochemical Journal*, vol. 376, no. 2, pp. 537–544, 2003.

[112] T. Hagen, C. T. Taylor, F. Lam, and S. Moncada, "Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1α," *Science*, vol. 302, no. 5652, pp. 1975–1978, 2003.

[113] R. Chowdhury, E. Flashman, J. Mecinović et al., "Studies on the reaction of nitric oxide with the hypoxia-inducible factor prolyl hydroxylase domain 2 (EGLN1)," *Journal of Molecular Biology*, vol. 410, no. 2, pp. 268–279, 2011.

[114] E. R. Block, H. Herrera, and M. Couch, "Hypoxia inhibits L-arginine uptake by pulmonary artery endothelial cells," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 269, no. 5, pp. L574–L580, 1995.

[115] R. Maas, "Pharmacotherapies and their influence on asymmetric dimethylargine (ADMA)," *Vascular Medicine*, vol. 10, no. 2, supplement, pp. S49–S57, 2005.

[116] J. Murray-Rust, J. Leiper, M. McAlister et al., "Structural insights into the hydrolysis of cellular nitric oxide synthase inhibitors by dimethylarginine dimethylaminohydrolase," *Nature Structural & Molecular Biology*, vol. 8, pp. 679–683, 2001.

[117] J. Leiper, J. Murray-Rust, N. McDonald, and P. Vallance, "S-nitrosylation of dimethylarginine dimethylaminohydrolase regulates enzyme activity: further interactions between nitric oxide synthase and dimethylarginine dimethylaminohydrolase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 21, pp. 13527–13532, 2002.

[118] A. Bakr, O. Pak, A. Taye et al., "Effects of dimethylarginine dimethylaminohydrolase-1 overexpression on the response of the pulmonary vasculature to hypoxia," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 49, pp. 491–500, 2013.

[119] A. Ito, P. S. Tsao, S. Adimoolam, M. Kimoto, T. Ogawa, and J. P. Cooke, "Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase," *Circulation*, vol. 99, no. 24, pp. 3092–3095, 1999.

[120] K. Y. Lin, A. Ito, T. Asagami et al., "Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase," *Circulation*, vol. 106, no. 8, pp. 987–992, 2002.

[121] K. Sydow and T. Münzel, "ADMA and oxidative stress," *Atherosclerosis Supplements*, vol. 4, no. 4, pp. 41–51, 2003.

[122] C. Y. Ivashchenko, B. T. Bradley, Z. Ao, J. Leiper, P. Vallance, and D. G. Johns, "Regulation of the ADMA-DDAH system in endothelial cells: a novel mechanism for the sterol response element binding proteins, SREBP1c and -2," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 298, no. 1, pp. H251–H258, 2010.

[123] K. Hasegawa, S. Wakino, T. Tanaka et al., "Dimethylarginine dimethylaminohydrolase 2 increases vascular endothelial growth factor expression through Sp1 transcription factor in endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 7, pp. 1488–1494, 2006.

[124] C. H. Jung, W. J. Lee, J. Y. Hwang et al., "Vaspin increases nitric oxide bioavailability through the reduction of asymmetric dimethylarginine in vascular endothelial cells," *PLoS ONE*, vol. 7, Article ID e52346, 2012.

[125] J. W. Eikelboom, E. Lonn, J. Genest Jr., G. Hankey, and S. Yusuf, "Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence," *Annals of Internal Medicine*, vol. 131, no. 5, pp. 363–375, 1999.

[126] J.-G. Zhang, J.-X. Liu, Z.-H. Li, L.-Z. Wang, Y.-D. Jiang, and S.-R. Wang, "Dysfunction of endothelial NO system originated from homocysteine-induced aberrant methylation pattern in promoter region of DDAH2 gene," *Chinese Medical Journal*, vol. 120, no. 23, pp. 2132–2137, 2007.

[127] W. Janssen, S. S. Pullamsetti, J. Cooke, N. Weissmann, A. Guenther, and R. T. Schermuly, "The role of dimethylarginine dimethylaminohydrolase (DDAH) in pulmonary fibrosis," *The Journal of Pathology*, vol. 229, pp. 242–249, 2013.

[128] M. Nandi, P. Kelly, B. Torondel et al., "Genetic and pharmacological inhibition of dimethylarginine dimethylaminohydrolase 1 is protective in endotoxic shock," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, pp. 2589–2597, 2012.

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Research Article

Contribution of Ca^{2+}-Dependent Cl^- Channels to Norepinephrine-Induced Contraction of Femoral Artery Is Replaced by Increasing EDCF Contribution during Ageing

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The activation of Ca^{2+}-dependent Cl^- channels during norepinephrine-induced contraction of vascular smooth muscle was suggested to depolarize cell membrane and to increase Ca^{2+} entry. Hypertension and ageing are associated with altered Ca^{2+} handling including possible activation of Ca^{2+}-dependent Cl^- channels. Our study was aimed to determine Ca^{2+}-dependent Cl^- channels contribution to norepinephrine-induced contraction during hypertension and ageing. Norepinephrine-induced concentration-response curves of femoral arteries from 6- and 12-month-old spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were recorded using wire myograph. Pretreatment with Ca^{2+}-dependent Cl- channel inhibitor indanyloxyacetic acid 94 [R(+)-IAA-94](IAA) attenuated norepinephrine-induced contraction in all groups, but relatively more in WKY than SHR arteries. The attenuation of norepinephrine-induced contraction after Ca^{2+}-dependent Cl^- channels blockade was partially reduced in 12-month-old WKY rats, but substantially diminished in 12-month-old SHR. IAA effect was enhanced after NO synthase inhibition but decreased by ageing. In 20-month-old WKY rats norepinephrine-induced contraction was not affected by IAA but was almost abolished after cyclooxygenase inhibition by indomethacin or niflumic acid. In conclusion, contribution of Ca^{2+}-dependent Cl^- channels to norepinephrine-induced contraction diminished with age, hypertension development, and/or NO synthesis inhibition. Ca^{2+}-dependent Cl^- channels are important for maintenance of normal vascular tone while their inactivation/closing might be a pathological mechanism.

1. Introduction

The stimulation of vascular smooth muscle cells with norepinephrine leads to the release of Ca^{2+} from intracellular Ca^{2+} stores through IP_3 pathway (phasic contraction) and thereafter to Ca^{2+} influx through the opening of L-type voltage-dependent Ca^{2+} channels (L-VDCC) (tonic contraction). The link between the opening of Ca^{2+} stores and L-VDCC could be mediated either through the elevation of intracellular Ca^{2+} $[Ca^{2+}]_i$ levels or might involve other mechanisms such as opening of Cl^- channels or closure of K^+ channels [1–3]. Brayden and Nelson [4] showed that pressure-induced contraction of vascular smooth muscle is mediated by Ca^{2+} influx through L-VDCC and is modulated by a negative feedback pathway involving activation of large-conductance Ca^{2+}-dependent K^+ channels. We demonstrated that these K^+ channels play an important role in modulating vascular contraction in spontaneous hypertension [5]. Calculated equilibrium potential for Cl^- in vascular cells is −26 mV [6], which is high enough to activate L-VDCC [7]. Furthermore, Ca^{2+}-dependent Cl^- channels play a role in Ca^{2+} uptake to sarcoplasmic reticulum in smooth muscle because the administration of Cl^- channel blockers NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) and IAA (indanyloxyacetic acid 94 (R(+)-IAA-94) almost completely blocked the Ca^{2+} uptake [8]. It is evident that Cl^- conductance is activated by the enhancement of either Ca^{2+} release from Ca^{2+} stores or Ca^{2+} influx [9]. The Ca^{2+}-dependent Cl^- channels exist in two states, open and closed, with a relatively long mean open time. Some of the agents that inhibit Ca^{2+}-dependent

Cl^- channels interact directly with the open channel, which suggests that the most likely role of Ca^{2+}-dependent Cl^- channels in smooth muscle is to produce membrane depolarization and contraction to neurotransmitters [10]. Therefore norepinephrine-induced contraction, which involves the increase of both Ca^{2+} release from Ca^{2+} stores and Ca^{2+} influx should be altered by the blockade of Ca^{2+}-dependent Cl^- channels. It has been shown that Ca^{2+}-dependent Cl^- channels play a major role in the response to norepinephrine in the portal vein [9] and mesenteric vein [11] or in the response of coronary and mesenteric arteries to endothelin [12]. Chloride channel blockers markedly attenuate both rapid and sustained responses of angiotensin II-induced contraction of renal vascular smooth muscle cells and thus contribute to, rather than being the consequence of, the initial rapid contractile response [13]. Pacaud et al. [14] suggested that the activation of Cl^- channels by Ca^{2+} release from intracellular stores might determine the maximal contraction by influencing the magnitude of membrane depolarization during agonist-induced contraction.

The activation of Ca^{2+}-dependent Cl^- channels in aortic smooth muscle cells occurs after caffeine-induced Ca^{2+} release without Ca^{2+} influx, but the repetitive activation of Cl^- channels depends on Ca^{2+} influx through L-VDCC. Moreover, the progressive disappearance of caffeine-induced Ca^{2+} release and Ca^{2+}-dependent Cl^- currents in smooth muscle cells treated with NO donors could result from NO-induced inhibition of ryanodine- and caffeine-sensitive Ca^{2+} channels and/or L-VDCC [15]. This mechanism could be more important for L-NAME-induced contraction of rat coronary arteries, because the changes of Cl^- conductance are involved in the L-NAME-induced contraction and the blockade of Cl^- channels abolished the L-NAME-induced contractions [16]. On the other hand, there are still confusing results about the involvement of endothelium in the effect of Cl^- channel activation. Using small mesenteric arteries Matchkov et al. [17] and Boedtkjer et al. [18] showed that there is a Cl^- current which is dependent on Ca^{2+} and cGMP and that the NO is necessary for the activation of this current, which leads to vasomotion and can participate in smooth muscle cell synchronization. This Ca^{2+}-dependent and cGMP-dependent Cl^- current is sensitive to Zn^{2+} ions but less sensitive to conventional Cl^- channel blockers.

There are numerous reports on altered vascular function in hypertension and/or ageing in rats (for review see [19]). These alterations involve impaired endothelium-dependent vascular relaxation due to the attenuated vasodilation induced by nitric oxide [20, 21], β-adrenoceptor stimulation [22, 23], changes in Ca^{2+} entry and cell Ca^{2+} handling [24, 25], and so forth. Prominent role of Ca^{2+} influx through L-VDCC in the control of vascular tone and blood pressure can be demonstrated both under the *in vivo* and *in vitro* conditions as we have previously reported in conscious SHR and their isolated femoral arteries [26–28].

Our aim was to quantify the changes in Ca^{2+}-dependent Cl^- channel-sensitive component of norepinephrine-induced contraction during the ageing and hypertension. Our hypothesis was that norepinephrine-stimulation of vascular smooth muscle opens/activates Ca^{2+}-dependent Cl^- channels in SHR vessels to a larger extent than in those of WKY. The NO synthase inhibition was used to augment the magnitude of norepinephrine-induced contraction as well as to modify the contribution of the above ion channels to this contraction. IAA was used to inhibit Ca^{2+}-dependent Cl^- channels in femoral arteries isolated from four groups of rats—adult WKY rats, aged WKY rats, adult SHR, and aged SHR. The second aim of our study was to compare the contribution of Ca^{2+}-dependent Cl^- channels and endothelium-derived constricting factor (EDCF) to norepinephrine-induced contraction (including their age-dependent changes). This was possible to examine only in femoral arteries because EDCF is produced in femoral but not in small mesenteric arteries [29]. We investigated femoral arteries of 20-month-old WKY rats after the blockade of Ca^{2+}-dependent Cl^- channels by IAA and after the inhibition of cyclooxygenase by indomethacin, which is the main enzyme producing endothelium-derived constricting factor (EDCF).

2. Material and Methods

2.1. General Procedure. Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) were sacrificed at the age of 6 months (adult WKY rats and adult SHR) and 12 months (aged WKY rats and aged SHR rats). The second series of experiments was carried out in 20-month-old WKY rats. Animals were housed under standard laboratory conditions (temperature $23 \pm 1^\circ$C, 12 h light-dark cycle, pelleted ST-1 diet, and tap water *ad libitum*). All procedures and experimental protocols, which were approved by the *Ethical Committee of the Institute of Physiology, Academy of Sciences of Czech Republic*, conform to the *European Convention on Animal Protection* and *Guidelines on Research Animal Use*.

Animals were anesthetized with ether and blood pressure was measured directly by the puncture of carotid artery. The animals were killed by overdose of CO_2 and after decapitation femoral arteries with intact endothelium were cut into 2 mm long segments and placed in a Mulvany-Halpern isometric myograph (M 510A, DMT, Denmark). The myograph chamber was filled with modified Krebs-Henseleit solution (119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 2.5 mM $CaCl_2$, 2 g/L glucose, 37°C) and bubbled with 95% O_2 and 5% CO_2. The inner arterial diameter was set to be 90% of the diameter predicted for the pressure of 100 mm Hg in the wire myograph. During 30 min of stabilization the vessels were left to achieve their basal tone. To provide the maximal depolarization-induced contraction vessels were incubated with a depolarizing solution (modified Krebs-Henseleit solution with 124 mM K^+ but without Na^+).

2.2. Experimental Protocol: The Role of Ca^{2+}-Dependent Cl^- Channel in Norepinephrine-Induced Contraction. The femoral arteries isolated from adult and aged WKY rats and SHR were studied in the first part of our experiments. Subsequent arterial contractions were induced by cumulative doses of norepinephrine (3.10^{-8} to 10^{-4} mol/L)

and then acetylcholine-induced relaxations (3.10^{-8} mol/L and 10^{-6} mol/L or 3.10^{-6} mol/L) of norepinephrine-precontracted vessels were measured. One segment with preserved endothelium was studied in each rat. After repeated washing and stabilization of basal tone norepinephrine-induced concentration-response curves were again determined in the same artery segment in the presence of NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 10^{-4} mol/L, preincubation time 15 min) or in the presence of Ca^{2+}-dependent Cl^{-} channel blocker indanyloxyacetic acid 94 (R(+)-IAA-94, IAA, 10^{-5} mol/L, preincubation time 10 min). Finally, these concentration-response curves were measured in the presence of both inhibitors L-NNA and IAA.

2.3. Experimental Protocol: The Changes of Ca^{2+}-Dependent Cl^{-} Channels and EDCF during Ageing. In the second part of our study we used femoral arteries isolated from 20-month-old WKY rats. Arterial contraction induced by cumulative doses of norepinephrine (10^{-8} to 10^{-4} mol/L) followed by arterial relaxation elicited by acetylcholine application (10^{-8} mol/L and 10^{-6} mol/L) were first measured in the absence of inhibitors. Thereafter norepinephrine-induced concentration-response curves were determined in the presence of Ca^{2+}-dependent Cl^{-} channel blocker indanyloxyacetic acid 94 (R(+)-IAA-94, IAA, 10^{-5} mol/L, preincubation time 10 min) and after 30 min washing period again in the presence of cyclooxygenase inhibitor indomethacin (IME, 10^{-5} mol/L, preincubation time 10 min). In another group of vessels norepinephrine-induced concentration-response curves were determined in the presence of niflumic acid (NIFLU, 10^{-5} mol/L, preincubation time 10 min) which was used as cyclooxygenase inhibitor and Ca^{2+}-dependent Cl^{-} channel blocker.

2.4. Drugs and Statistical Analysis. All chemicals were obtained from Sigma (Heidelberg, Germany). R(+)-IAA-94 and niflumic acid were dissolved in ethanol maintaining the final concentration of ethanol in the chamber under 1%. Indomethacin was dissolved in 170 mM Na_2CO_3. Logistic model was used for fitting concentration-response curves through the measure values [30]. Pharmacodynamic characteristics—EC50 (half-maximal effective concentration, log mol/L), E_{max} (maximal contraction, mN/mm), and slope—were calculated for each norepinephrine-induced concentration-response curve. Data are presented as mean ± S.E.M. Statistical analysis was performed with one-way ANOVA and *post hoc* least significant difference test.

3. Results

3.1. Basal Characteristics. Blood pressure was higher in SHR than WKY rats without any significant age-dependent increase. The inner diameter of studied femoral arteries was always significantly smaller in SHR than in WKY rats, but it increased with age in both rat strains (Table 1).

3.2. Vascular Responses of Femoral Arteries in Adult and Aged WKY or SHR. The administration of cumulative norepinephrine concentrations to isolated femoral arteries yielded concentration-response curves which were similar in vessels from adult WKY and aged WKY. As far as concentration-response curves of SHR arteries are concerned, the maximal contraction (E_{max}) was enhanced in vessels from SHR compared to WKY vessels. Ageing increased the maximal contraction in vessels from SHR only (Figure 1; Table 2). Acetylcholine at the concentration 3.10^{-8} mol/L relaxed the arteries of adult and aged WKY as well as those of adult SHR by about 50%. The vessels from aged SHR relaxed by 37% only (Table 3). At the higher concentrations of acetylcholine (10^{-6} mol/L in WKY and 3.10^{-6} mol/L in SHR) the maximal acetylcholine-induced relaxation of isolated femoral arteries was only slightly attenuated in SHR arteries (Table 3). The wall tension decrease after acetylcholine was greater in SHR vessels, which corresponds to the enhanced arterial contraction in SHR.

3.3. Effect of Ca^{2+}-Dependent Cl^{-} Channels Inhibition on Norepinephrine-Induced Concentration-Response Curves. The inhibition of Ca^{2+}-dependent Cl^{-} channels with IAA almost abolished the norepinephrine-induced contraction of vessels from adult and aged WKY rats and largely reduced the contraction of vessels from adult SHR. The relative inhibitory effect of IAA was surprisingly smallest in femoral arteries isolated from aged SHR (Figure 1; Table 2). The relative contribution of Ca^{2+}-dependent Cl^{-} channel-sensitive component to norepinephrine-induced contraction was 97% in adult WKY, 90% in aged WKY, 80% in adult SHR, but only 55% in aged SHR. The sensitivity to norepinephrine (EC50) was enhanced and E_{max} was impaired by IAA pretreatment of WKY vessels compared to the control conditions. In contrast, there were no significant changes in norepinephrine sensitivity of concentration-response curves in SHR vessels (Table 2).

3.4. Effect of Ca^{2+}-Dependent Cl^{-} Channels Inhibition on Norepinephrine-Induced Concentration-Response Curves during NO Synthase Inhibition. The inhibition of NO synthase with L-NNA shifted the concentration-response curves to the left in all groups (Figure 1). E_{max} was substantially increased as compared to the control conditions (Table 2). In the absence of NO synthesis the inhibition of Ca^{2+}-dependent Cl^{-} channels shifted norepinephrine-induced concentration-response curves to the right (Figure 1) and reduced E_{max} more in WKY than in SHR vessels (Table 2). The relative contribution of Ca^{2+}-dependent Cl^{-} channel-sensitive component represented 93% in adult WKY and 78% in aged WKY,but only 63% in adult SHR and 28% of norepinephrine-induced contraction in aged SHR. Thus, the effect of IAA was again smallest in aged SHR.

3.5. The Changes between Ca^{2+}-Dependent Cl^{-} Channels and EDCF during Ageing. Norepinephrine-induced contraction of vessels isolated from 20-month-old WKY rats were slightly more sensitive to norepinephrine and reached slightly higher maximal contraction values as compared to adult and aged WKY rats. The acetylcholine-induced relaxation was impaired at low concentration of acetylcholine, but it was not

Table 1: Blood pressure and the diameter of isolated femoral arteries in adult and aged Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR).

Parameters	Adult WKY (6 months) ($n = 6$)	Aged WKY (12 months) ($n = 7$)	Adult SHR (6 months) ($n = 6$)	Aged SHR (12 months) ($n = 9$)
SBP (mm Hg)	131 ± 3	134 ± 4	221 ± 5[a]	215 ± 6[c]
MAP (mm Hg)	109 ± 3	112 ± 3	182 ± 5[a]	187 ± 4[c]
DBP (mmHg)	91 ± 3	94 ± 3	152 ± 4[a]	163 ± 4[c]
Diameter (μm)	995 ± 21	1052 ± 20[a]	843 ± 27[a]	943 ± 17[b,c]

Data are presented as mean ± S.E.M. SBP: systolic blood pressure; MAP: mean arterial pressure; DBP: diastolic blood pressure. Significant differences: [a]$P < 0.05$ versus adult WKY; [b]$P < 0.05$ versus adult SHR; [c]$P < 0.05$ versus aged WKY.

changed at higher acetylcholine concentration and reached 79 ± 2% of norepinephrine precontraction (Table 3).

Surprisingly, the application of Ca^{2+}-dependent Cl^- channel blocker IAA did not impair the norepinephrine-induced contraction as it was shown in our previous experiments with 6- and 12-month-old rats. As in previous experiments with 6- and 12-month-old rats IAA increased EC50, but the E_{max} was not altered significantly. The subsequent inhibition of cyclooxygenase with indomethacin almost fully abolished the norepinephrine-induced contraction. In parallel experiments the application of niflumic acid, which is considered to be Ca^{2+}-dependent Cl^- channel blocker and cyclooxygenase inhibitor, also abolished the norepinephrine-induced contraction to a similar extent as indomethacin. Our experiments have clearly shown that niflumic acid is a potent cyclooxygenase inhibitor, which cannot be used as a simple Ca^{2+}-dependent Cl^- channel blocker (Table 4; Figure 2).

4. Discussion

We have investigated the effect of ageing and hypertension on the participation of Ca^{2+}-dependent Cl^- channels in norepinephrine-induced contraction of femoral arteries. When evaluating Ca^{2+}-dependent Cl^- channel-sensitive component of norepinephrine-induced contraction we have demonstrated that the inhibition of Ca^{2+}-dependent Cl^- channels largely impaired the norepinephrine-induced contraction of femoral arteries in all studied groups of rats. Thus, our data are in good agreement with the earlier findings on norepinephrine-induced increase of Cl^- conductance in portal vein smooth muscle cells [9]. However, the relative effects were greatest in the arteries of adult WKY rats but smallest in vessels of aged SHR. Our results showed that the contribution of Ca^{2+}-dependent Cl^- channels to norepinephrine-induced contraction is reduced during ageing, and hypertension further enhanced this reduction. The mechanism(s) responsible for the residual part of norepinephrine-induced arterial contraction seen mainly in SHR vessels remains to be determined. Resting membrane potential of vascular smooth muscle cells of SHR is increasing during the hypertension development and stays significantly higher compared to normotensive WKY rats [31]. Contrary to our expectations, norepinephrine stimulation of vascular smooth muscle did not open/activate Ca^{2+}-dependent Cl^- channels to a larger extent in vascular smooth muscle of SHR.

IAA is a potent Ca^{2+}-dependent Cl^- channel inhibitor, but it has also a relatively high affinity to L-VDCC [32]. It is unlikely that IAA affected the L-VDCC significantly in our experiments because a greater IAA effect was disclosed in vessels from adult WKY compared to those from SHR, although L-VDCC plays a more important role in SHR than in WKY [24]. Ca^{2+}-dependent Cl^- channels blockers also activate large-conductance Ca^{2+}-activated K^+ channels [33]. The concentration used in our experiments was lower as assumed for the activation of Ca^{2+}-activated K^+ channels (10^{-5} mol/L versus 5×10^{-4} mol/L, [34]). To our knowledge there is no evidence about decreasing influence of Ca^{2+}-activated K^+ channels during ageing and further experiments are needed to show if the effect of IAA seen in our study was partially through Ca^{2+}-activated K^+ channels.Niflumic acid is also a potent cyclooxygenase inhibitor. A comparison of the effect of indomethacin and niflumic acid clearly shows that both compounds are acting through the inhibition of cyclooxygenase pathway because it was impossible to produce additive changes in contraction by the combination of these two compounds.

We have recently demonstrated [35] that a considerable part of norepinephrine-induced contraction of rat femoral artery was mediated by endothelium-derived constricting factor (EDCF). This type of arterial contraction can be largely prevented by the pretreatment of vessels with cyclooxygenase inhibitor indomethacin. Indomethacin administration was also capable of inducing a substantial reduction of the already developed arterial contraction. It is important to note that EDCF contribution to norepinephrine-induced arterial contraction was increasing with age and hypertension development [35]. The same was reported for the role of EDCF in the age-dependent impairment of acetylcholine-induced endothelium-dependent vascular relaxation in developing SHR [36, 37]. The mechanisms underlying the age-dependent increase of EDCF formation and/or action remain unclear. It can be speculated that the earlier described age-dependent elevation of intracellular Na^+ concentration could augment cyclooxygenase 2 expression as it was demonstrated in cells with increased $Na^+{}_i/K^+{}_i$ ratio [38]. Nevertheless, it should be mentioned that EDCF participation in vascular contraction elicited by norepinephrine or high doses of acetylcholine

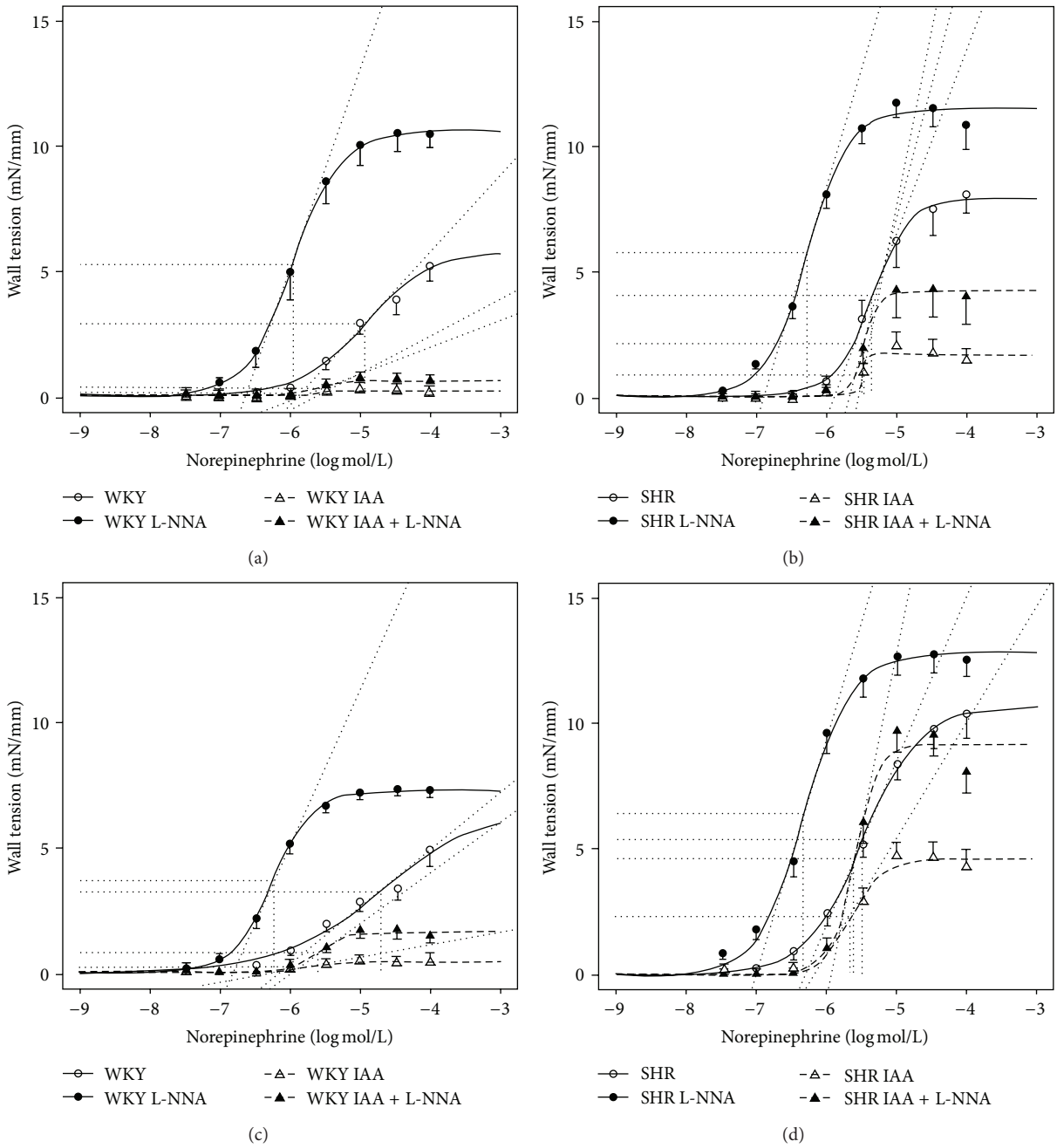

Figure 1: Norepinephrine concentration-response curves obtained in femoral arteries of adult WKY rats (6 months old, (a)), adult SHR (6 months old, (b)), aged WKY rats (12 months old, (c)), and aged SHR (12 months old, (d)) recorded under the control conditions and after the inhibition of NO synthase (L-NNA) or Ca^{2+}-dependent Cl^- channels blockade (R(+)-IAA-94, IAA) or both (IAA + L-NNA). Data are presented as mean ± S.E.M. (for number of vessels see Table 1). Depicted curves were calculated from average values obtained at studied norepinephrine concentrations.

is characteristic for conduit arteries because it is absent in resistance arteries such as small mesenteric vessels [29]. Thus the role of EDCF in blood pressure control remains questionable.

Ca^{2+}-dependent Cl^- channels are involved in a major part of norepinephrine-induced contraction of adult and aged WKY arteries, but the contribution of these channels to norepinephrine-induced contraction is decreasing with age and/or hypertension development as was shown using the arteries of 20-month-old WKY rats in which the blockade of Ca^{2+}-dependent Cl^- channels was without effect on norepinephrine-induced contraction (Figure 2). This is in contrast with the increasing role of cyclooxygenase-sensitive component of norepinephrine-induced contraction during ageing and hypertension [35]. Thus the participation of Cl^- channels in norepinephrine-induced arterial

Table 2: Pharmacodynamic parameters of norepinephrine concentration-response curves determined in individual femoral arteries isolated from adult and aged WKY and SHR which were studied under the control conditions (CONTROL), in the presence of N^{ω}-nitro-L-arginine (L-NNA, NO synthase inhibitor) and in the presence of R(+)-IAA-94 (IAA, Ca^{2+}-dependent Cl^{-} channel blocker) as well as in the presence of IAA and L-NNA (IAA + L-NNA).

	Parameters	Adult WKY (6 months)	Aged WKY (12 months)	Adult SHR (6 months)	Aged SHR (12 months)
CONTROL (6/7/6/9)	EC50 (log mol/L)	-4.93 ± 0.09	-5.11 ± 0.13	-5.12 ± 0.15	-5.43 ± 0.08
	E_{max} (mN/mm)	5.26 ± 0.84	5.30 ± 0.64	8.25 ± 0.32^{a}	$10.41 \pm 0.70^{b,c}$
	Slope	3.69 ± 0.55	3.11 ± 0.77	11.27 ± 0.52^{a}	8.80 ± 1.10^{c}
L-NNA (6/7/6/7)	EC50 (log mol/L)	$-5.84 \pm 0.13^{d,e}$	$-6.14 \pm 0.07^{d,e}$	$-6.17 \pm 0.04^{d,e}$	$-6.28 \pm 0.07^{d,e}$
	E_{max} (mN/mm)	$10.48 \pm 0.70^{d,e}$	$7.29 \pm 0.30^{a,d,e}$	$11.31 \pm 0.54^{d,e}$	$12.63 \pm 0.78^{c,d,e}$
	Slope	10.03 ± 1.60^{d}	9.77 ± 1.84	12.24 ± 0.46	15.71 ± 3.12
IAA (6/7/6/9)	EC50 (log mol/L)	-5.53 ± 0.13^{d}	$-5.79 \pm 0.13^{d,e}$	-5.35 ± 0.05	-5.48 ± 0.07
	E_{max} (mN/mm)	0.15 ± 0.05^{d}	0.53 ± 0.08^{d}	$1.68 \pm 0.20^{a,d,e}$	$4.66 \pm 0.60^{b,c,d,e}$
	Slope	2.63 ± 0.78	1.68 ± 0.12	9.50 ± 1.96	21.12 ± 7.04
IAA + L-NNA (6/7/6/7)	EC50 (log mol/L)	-5.42 ± 0.12^{d}	-5.47 ± 0.08^{d}	-5.28 ± 0.02	-5.46 ± 0.07^{b}
	E_{max} (mN/mm)	0.76 ± 0.03^{d}	1.64 ± 0.38^{d}	$4.21 \pm 0.52^{a,d}$	$9.17 \pm 0.90^{b,c}$
	Slope	2.00 ± 0.77	6.29 ± 3.20	18.40 ± 4.49^{a}	14.03 ± 0.76

Data are presented as mean ± S.E.M., the number of vessels studied under different experimental conditions is indicated in parentheses (adult WKY/aged WKY/adult SHR/aged SHR). Significant differences: $^{a}P < 0.05$ versus adult WKY; $^{b}P < 0.05$ versus adult SHR; $^{c}P < 0.05$ versus aged WKY; $^{d}P < 0.05$ versus Control; $^{e}P < 0.05$ versus IAA + L-NNA.

Table 3: Maximal norepinephrine-induced contraction and acetylcholine-induced relaxation of femoral arteries isolated from adult and aged WKY and SHR.

Parameters	Adult WKY (6 months) ($n = 6$)	Aged WKY (12 months) ($n = 7$)	Adult SHR (6 months) ($n = 6$)	Aged SHR (12 months) ($n = 9$)	WKY (20 months) ($n = 16$)
Norepinephrine-induced (10^{-4} mol/L) maximal contraction (mN/mm)	5.26 ± 0.84	5.30 ± 0.64	8.25 ± 0.32^{a}	$10.41 \pm 0.70^{a,b}$	6.79 ± 0.20
Acetylcholine-induced (3.10^{-8} mol/L) relaxation (%)	53 ± 3	47 ± 6	55 ± 4	$37 \pm 4^{a,b}$	18 ± 4^{c}
Acetylcholine-induced (3.10^{-8} mol/L) wall tension decrease (mN/mm)	-2.70 ± 0.36	-2.02 ± 0.41	$-4.37 \pm 0.70^{(a)}$	-4.18 ± 0.55^{a}	-0.98 ± 0.22^{c}
Acetylcholine-induced relaxation (%)*	85 ± 4	82 ± 5	67 ± 4^{a}	70 ± 2	79 ± 2
Acetylcholine-induced wall tension decrease (mN/mm)*	-4.37 ± 0.65	-3.49 ± 0.44	-5.30 ± 0.38	$-8.04 \pm 0.84^{a,b}$	-4.70 ± 0.23

Data are presented as mean ± S.E.M.; $^{a}P < 0.05$ versus aged-matched WKY; $^{b}P < 0.05$ versus adult SHR; $^{c}P < 0.05$ versus WKY; $^{(a)}$represents borderline significance $P < 0.08$. *Maximal acetylcholine-induced relaxations were achieved in femoral arteries of WKY at the concentration 10^{-6} mol/L and in femoral arteries of SHR at the concentration 3.10^{-6} mol/L.

contraction was drastically reduced in aged SHR compared to adult animals, but it was not found in arteries of 20-month-old WKY rats. It seems that the role of Ca^{2+}-dependent Cl^{-} channels is replaced by the increasing influence of EDCF (cyclooxygenase-sensitive component of norepinephrine-induced contraction). It remains to be determined which other cyclooxygenase-sensitive mechanism is responsible for the progressive age-dependent augmentation of norepinephrine-induced contraction of large arteries in ageing and hypertension.

In contrast to the lack of effects of Ca^{2+}-dependent Cl^{-} channel inhibition, the inhibition of cyclooxygenase with indomethacin led to the attenuation of norepinephrine-induced contraction of femoral arteries of 20-month-old WKY rats. In parallel experiments with vessels of these old rats, the application of niflumic acid led to the attenuation of norepinephrine-induced contraction as well. Niflumic acid is considered to be a Ca^{2+}-dependent Cl^{-} channel inhibitor as well as cyclooxygenase inhibitor. Our experiments clearly indicated that niflumic acid is a potent cyclooxygenase inhibitor and that the results obtained with niflumic acid in other studies should be considered carefully. Unfortunately, we were not able to investigate the effect of niflumic acid after the administration of indomethacin on norepinephrine-induced contraction because indomethacin alone almost abolished the contraction to norepinephrine.

The inhibition of NO synthesis pronounced the age- and hypertension-dependent reduction of Ca^{2+}-dependent Cl^{-} channel-sensitive component of norepinephrine-induced contraction. It is possible that the endothelial factors (such as NO) might contribute to the reduction of IAA-sensitive component of norepinephrine-induced contraction. Further

Table 4: Pharmacodynamic parameters of norepinephrine concentration-response curves determined in individual femoral arteries isolated from 20-month-old WKY rats in the presence of R(+)-IAA-94 (IAA, Ca^{2+}-dependent Cl^- channel blocker) and indomethacin (IME, cyclooxygenase inhibitor) as well as in the presence of niflumic acid (NIFLU, cyclooxygenase inhibitor, and Cl^- channel blocker).

	Parameters	Control ($n = 16$)	IAA ($n = 8$)	IME ($n = 8$)	NIFLU ($n = 8$)
WKY	EC50 (log mol/L)	-5.22 ± 0.12	-5.89 ± 0.10^{a}	-4.67 ± 0.18^{b}	-4.93 ± 0.18^{b}
	E_{max} (mN/mm)	6.79 ± 0.20	6.86 ± 0.26	$1.40 \pm 0.10^{a,b}$	$1.18 \pm 0.06^{a,b}$
	Slope	3.90 ± 0.37	4.49 ± 0.35	$0.43 \pm 0.10^{a,b}$	$0.63 \pm 0.09^{a,b}$

Data are presented as mean ± S.E.M.; $^{a}P < 0.05$ versus control; $^{b}P < 0.05$ versus IAA.

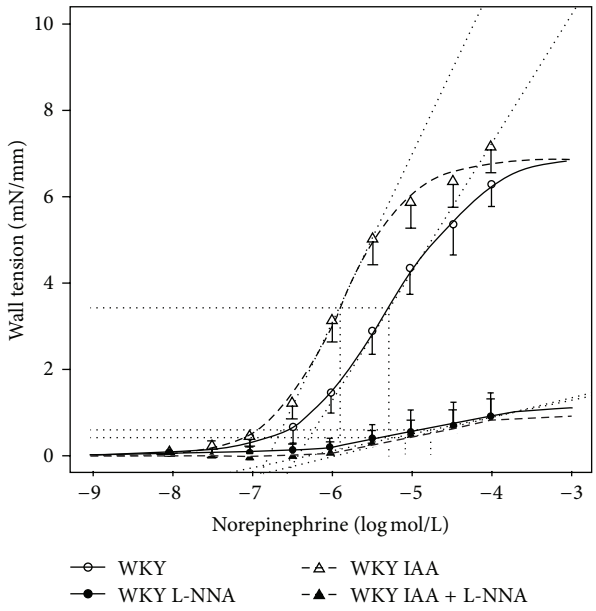

Figure 2: Norepinephrine concentration-response curves obtained in femoral arteries of 20-month-old WKY rats (WKY) recorded under the control conditions and after Ca^{2+}-dependent Cl^- channels blockade (R(+)-IAA-94, IAA) or after the inhibition of cyclooxygenase (indomethacin, IME) or in the presence of niflumic acid (NIFLU). Data are presented as mean ± S.E.M. (for number of vessels see Table 4). Depicted curves were calculated from average values obtained at studied norepinephrine concentrations.

studies with more specific compounds are needed to separate the Cl^- current dependent on NO as shown by Matchkov et al. [17] and Boedtkjer et al. [18] from the Ca^{2+}-dependent Cl^- current in vascular smooth muscle cells.

It is important to note that the inhibition of Ca^{2+}-dependent Cl^- channels was suggested as a possible therapeutic target in hypertension [39]. In the present study we provide the first evidence that the activation/opening of Ca^{2+}-dependent Cl^- channels is important for the maintenance of normal physiological state during norepinephrine-induced contraction, at least in femoral arteries. Further attention should be paid to the interactions of Cl^- channels with other ion transporter systems. Recent data indicate that Cl^- channels in vascular smooth muscle cells are under the control of inwardly directed Na^+, K^+, and $2Cl^-$ cotransport (NKCC1). Numerous research groups demonstrated that the inhibitors of this carrier such as bumetanide and furosemide sharply diminished contraction of blood vessels elicited by norepinephrine or other vasoconstrictors (for review see [40]). Increased *nkcc1* mRNA expression and NKCC1 protein content in the aorta of SHR was accompanied by hypomethylation of the *nkcc1* gene promoter [41]. It is important to note that the methylation of *nkcc1* promoter in normotensive WKY rats was increased with age, whereas in SHR it remained hypomethylated after hypertension development. Both increased *nkcc1* expression and inhibitory action of bumetanide on mesenteric artery contractions were increased with age in SHR but not in WKY rats [42]. It would therefore be desirable to study the effects of Ca^{2+}-dependent Cl^- channel blockers on norepinephrine-induced vascular contraction in the presence of NKCC1 inhbitors.

In conclusion, our study demonstrated a considerable contribution of Ca^{2+}-dependent Cl^- channels to norepinephrine-induced arterial contraction, which diminishes with age, hypertension development, and/or inhibition of NO synthesis. Further studies are needed to provide the evidence for our hypothesis that Ca^{2+}-dependent Cl^- channels are important for the maintenance of normal physiological state in the vascular system. The loss or inactivation of these channels during ageing and/or hypertension development could be seen as a pathological mechanism.

5. Limitations of the Study

The contribution of Ca^{2+}-dependent Cl^- channels in norepinephrine-induced arterial contraction should be investigated in young normotensive and genetically hypertensive rats to reveal their potential role in the development of hypertension. The contribution of these ion channels to arterial contraction elicited by other vasoconstrictors (such as angiotensin II or endothelin-1) should also be studied. It would be desirable to evaluate the above mechanism in other forms of experimental hypertension.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] A. P. Somlyo and B. Himpens, "Cell calcium and its regulation in smooth muscle," *FASEB Journal*, vol. 3, no. 11, pp. 2266–2276, 1989.

[2] H. Karaki, H. Ozaki, M. Hori et al., "Calcium movements, distribution, and functions in smooth muscle," *Pharmacological Reviews*, vol. 49, no. 2, pp. 157–230, 1997.

[3] M. J. Berridge, "Smooth muscle cell calcium activation mechanisms," *Journal of Physiology*, vol. 586, no. 21, pp. 5047–5061, 2008.

[4] J. E. Brayden and M. T. Nelson, "Regulation of arterial tone by activation of calcium-dependent potassium channels," *Science*, vol. 256, no. 5056, pp. 532–535, 1992.

[5] S. Líšková, M. Petrová, P. Karen, J. Kuneš, and J. Zicha, "Influence of calcium-dependent potassium channel blockade and nitric oxide inhibition on norepinephrine-induced contractions in two forms of genetic hypertension," *Journal of the American Society of Hypertension*, vol. 4, no. 3, pp. 128–134, 2010.

[6] R. Casteels, K. Kitamura, H. Kuriyama, and H. Suzuki, "The membrane properties of the smooth muscle cells of the rabbit main pulmonary artery," *Journal of Physiology*, vol. 271, no. 1, pp. 41–61, 1977.

[7] K. Kitamura and J. Yamazaki, "Chloride channels and their functional roles in smooth muscle tone in the vasculature," *Japanese Journal of Pharmacology*, vol. 85, no. 4, pp. 351–357, 2001.

[8] N. S. Pollock, M. E. Kargacin, and G. J. Kargacin, "Chloride channel blockers inhibit Ca^{2+} uptake by the smooth muscle sarcoplasmic reticulum," *Biophysical Journal*, vol. 75, no. 4, pp. 1759–1766, 1998.

[9] P. Pacaud, G. Loirand, C. Mironneau, and J. Mironneau, "Noradrenaline activates a calcium-activated chloride conductance and increases the voltage-dependent calcium current in cultured single cells of rat portal vein," *British Journal of Pharmacology*, vol. 97, no. 1, pp. 139–146, 1989.

[10] W. A. Large and Q. Wang, "Characteristics and physiological role of the Ca^{2+}-activated Cl^- conductance in smooth muscle," *American Journal of Physiology*, vol. 271, no. 2, pp. C435–C454, 1996.

[11] D. F. Van Helden, "An α-adrenoceptor-mediated chloride conductance in mesenteric veins of the guinea-pig," *Journal of Physiology*, vol. 401, pp. 489–501, 1988.

[12] U. Klockner and G. Isenberg, "Endothelin depolarizes myocytes from porcine coronary and human mesenteric arteries through a Ca-activated chloride current," *Pflugers Archiv*, vol. 418, no. 1-2, pp. 168–175, 1991.

[13] A. J. Fuller, B. C. Hauschild, R. Gonzalez-Villalobos et al., "Calcium and chloride channel activation by angiotensin II-AT_1 receptors in preglomerular vascular smooth muscle cells," *American Journal of Physiology*, vol. 289, no. 4, pp. F760–F767, 2005.

[14] P. Pacaud, G. Loirand, A. Baron, C. Mironneau, and J. Mironneau, "Ca^{2+} channel activation and membrane depolarization mediated by Cl^- channels in response to noradrenaline in vascular myocytes," *British Journal of Pharmacology*, vol. 104, no. 4, pp. 1000–1006, 1991.

[15] Y. Hirakawa, M. Gericke, R. A. Cohen, and V. M. Bolotina, "Ca^{2+}-dependent Cl^- channels in mouse and rabbit aortic smooth muscle cells: regulation by intracellular Ca^{2+} and NO," *American Journal of Physiology*, vol. 277, no. 5, pp. H1732–H1744, 1999.

[16] J. E. Graves, I. A. Greenwood, and W. A. Large, "Tonic regulation of vascular tone by nitric oxide and chloride ions in rat isolated small coronary arteries," *American Journal of Physiology*, vol. 279, no. 6, pp. H2604–H2611, 2000.

[17] V. V. Matchkov, C. Aalkjaer, and H. Nilsson, "A cyclic GMP-dependent calcium-activated chloride current in smooth-muscle cells from rat mesenteric resistance arteries," *Journal of General Physiology*, vol. 123, no. 2, pp. 121–134, 2004.

[18] D. M. B. Boedtkjer, V. V. Matchkov, E. Boedtkjer, H. Nilsson, and C. Aalkjaer, "Vasomotion has chloride-dependency in rat mesenteric small arteries," *Pflugers Archiv*, vol. 457, no. 2, pp. 389–404, 2008.

[19] M. Pintérová, J. Kuneš, and J. Zicha, "Altered neural and vascular mechanisms in hypertension," *Physiological Research*, vol. 60, pp. 381–402, 2011.

[20] Y. Shirasaki, C. Su, and T. J.-F. Lee, "Endothelial modulation of vascular relaxation to nitrovasodilators in aging and hypertension," *Journal of Pharmacology and Experimental Therapeutics*, vol. 239, no. 3, pp. 861–866, 1986.

[21] F. Li and I. G. Joshua, "Decreased arteriolar endothelium-derived relaxing factor production during the development of genetic hypertension," *Clinical and Experimental Hypertension*, vol. 15, no. 3, pp. 511–526, 1993.

[22] S. Fujimoto, Y. Dohi, and K. Aoki, "Diminished β-adrenoceptor-mediated relaxation of arteries from spontaneously hypertensive rats before and during development of hypertension," *European Journal of Pharmacology*, vol. 136, no. 2, pp. 179–187, 1987.

[23] S. Arribas, J. Marin, A. Ponte, G. Balfagon, and M. Salaices, "Norepinephrine-induced relaxations in rat aorta mediated by endothelial beta adrenoceptors. Impairment by ageing and hypertension," *Journal of Pharmacology and Experimental Therapeutics*, vol. 270, no. 2, pp. 520–527, 1994.

[24] N. J. Rusch and K. Hermsmeyer, "Calcium currents are altered in the vascular muscle cell membrane of spontaneously hypertensive rats," *Circulation Research*, vol. 63, no. 6, pp. 997–1002, 1988.

[25] R. H. Cox and I. M. Lozinskaya, "Augmented calcium currents in mesenteric artery branches of the spontaneously hypertensive rats," *Hypertension*, vol. 26, no. 6, pp. 1060–1064, 1995.

[26] L. Paulis, S. Líšková, M. Pintérová, Z. Dobešová, J. Kuneš, and J. Zicha, "Nifedipine-sensitive noradrenergic vasoconstriction is enhanced in spontaneously hypertensive rats: the influence of chronic captopril treatment," *Acta Physiologica*, vol. 191, pp. 255–266, 2007.

[27] M. Pintérová, S. Líšková, Z. Dobešová, M. Behuliak, J. Kuneš, and J. Zicha, "Impaired control of L-type voltage-dependent calcium channels in experimental hypertension," *Physiological Research*, vol. 58, supplement 2, pp. S43–S54, 2009.

[28] M. Pintérová, P. Karen, J. Kuneš, and J. Zicha, "Role of nifedipine-sensitive sympathetic vasoconstriction in maintenance of high blood pressure in spontaneously hypertensive rats: effect of Gi-protein inactivation by pertussis toxin," *Journal of Hypertension*, vol. 28, no. 5, pp. 969–978, 2010.

[29] L. Paulis, J. Zicha, J. Kunes et al., "Regression of L-NAME-induced hypertension: the role of nitric oxide and endothelium-derived constricting factor," *Hypertension Research*, vol. 31, no. 4, pp. 793–803, 2008.

[30] J. Janáček and I. Prášil, "Quantification of plant frost injury by nonlinear fitting of an S-shaped function," *Cryoletters*, vol. 12, pp. 47–52, 1991.

[31] D. W. Cheung, "Membrane potential of vascular smooth muscle and hypertension in spontaneously hypertensive rats," *Canadian Journal of Physiology and Pharmacology*, vol. 62, no. 8, pp. 957–960, 1984.

[32] J. M. Doughty, A. L. Miller, and P. D. Langton, "Non-specificity of chloride channel blockers in rat cerebral arteries: block of the L-type calcium channel," *Journal of Physiology*, vol. 507, no. 2, pp. 433–439, 1998.

[33] I. A. Greenwood and N. Leblanc, "Overlapping pharmacology of Ca^{2+}-activated Cl^- and K^+ channels," *Trends in Pharmacological Sciences*, vol. 28, no. 1, pp. 1–5, 2007.

[34] C. Toma, I. A. Greenwood, R. M. Helliwell, and W. A. Large, "Activation of potassium currents by inhibitors of calcium-activated chloride conductance in rabbit portal vein smooth muscle cells," *British Journal of Pharmacology*, vol. 118, no. 3, pp. 513–520, 1996.

[35] S. Líšková, M. Petrová, K. Petr, J. Kuneš, and J. Zicha, "Effects of aging and hypertension on the participation of endothelium-derived constricting factor (EDCF) in norepinephrine-induced contraction of rat femoral artery," *European Journal of Pharmacology*, vol. 667, no. 1–3, pp. 265–270, 2011.

[36] P. A. C. Watt and H. Thurston, "Endothelium-dependent relaxation in resistance vessels from the spontaneously hypertensive rats," *Journal of Hypertension*, vol. 7, no. 8, pp. 661–666, 1989.

[37] T. Koga, Y. Takata, K. Kobayashi, S. Takishita, Y. Yamashita, and M. Fujishima, "Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat," *Hypertension*, vol. 14, no. 5, pp. 542–548, 1989.

[38] S. V. Koltsova, Y. Trushina, M. Haloui et al., "Ubiquitous $[Na^+]_i/[K^+]_i$-sensitive transcriptome in mammalian cells: evidence for $Ca^{2+}{}_i$-independent excitation-transcription coupling," *PLoS ONE*, vol. 7, Article ID e38032, 2012.

[39] K. Goto, F. R. Edwards, and C. E. Hill, "Depolarization evoked by acetylcholine in mesenteric arteries of hypertensive rats attenuates endothelium-dependent hyperpolarizing factor," *Journal of Hypertension*, vol. 25, no. 2, pp. 345–359, 2007.

[40] S. N. Orlov, S. V. Koltsova, J. Tremblay, M. B. Baskakov, and P. Hamet, "NKCC1 and hypertension: role in the regulation of vascular smooth muscle contractions and myogenic tone," *Annals of Medicine*, vol. 44, supplement 1, pp. S111–S118, 2012.

[41] H.-A. Lee, I. Baek, Y. M. Seok et al., "Promoter hypomethylation upregulates Na^+-K^+-$2Cl^-$ cotransporter 1 in spontaneously hypertensive rats," *Biochemical and Biophysical Research Communications*, vol. 396, no. 2, pp. 252–257, 2010.

[42] H.-M. Cho, H.-A. Lee, H. Y. Kim, H. S. Han, and I. K. Kim, "Expression of Na^+-K^+-2Cl cotransporter 1 is epigenetically regulated during postnatal development of hypertension," *American Journal of Hypertension*, vol. 24, no. 12, pp. 1286–1293, 2011.

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Research Article

Endothelial Function in a Mouse Model of Myeloperoxidase Deficiency

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Myeloperoxidase (MPO) activity is suggested to reduce the function of vascular nitric oxide, thereby contributing to endothelial dysfunction, although data in rodents are inconclusive. We examined vascular contractile and relaxant responses in MPO-deficient ($MPO^{-/-}$) and wild-type mice to investigate the role for myeloperoxidase in the development of endothelial dysfunction. Carotid and saphenous arteries were taken from 8-month-old mice and studied in a myograph. Responses of carotid arteries to phenylephrine, high potassium, or acetylcholine (Ach) were statistically not different from controls. Treatment with lipopolysaccharide (LPS; to enhance endothelial dysfunction) reduced responses to Ach in $MPO^{-/-}$ but did not affect responses in wild-type. In response to high concentrations of Ach, carotid arteries responded with transient contractions, which were not different between the groups and not affected by LPS treatment. Saphenous arteries from $MPO^{-/-}$ had smaller normalized diameters and developed less contractile force. Vessels from $MPO^{-/-}$ were less sensitive to Ach than controls. These data suggest that mature MPO-deficient mice do not show enhanced endothelial function compared to wild-type mice, even when provoked with LPS treatment. The EDHF response appears to be reduced in MPO deficiency.

1. Introduction

Endothelium-dependent vasodilatation in response to, for example, elevations of blood flow is considered a hallmark of normal vascular function [1]. Reduced vasodilator capacity—endothelial dysfunction—has been associated with cardiovascular disease, in particular atherosclerosis [2]. Although atherosclerosis affects predominantly the large arteries, endothelial dysfunction is probably a general feature of the endothelium in most parts of the vasculature of the atherosclerotic patient [3].

Recently, attention has focused on the enzyme myeloperoxidase (MPO) as a possible contributor to endothelial dysfunction [4]. MPO catalyses the conversion of hydrogen peroxide to hypochlorous acid. This product may react with nitric oxide, creating peroxynitrite, which has detrimental effects on effector cell function. Hypochlorous acid also chlorinates arginine, thus consuming the substrate for nitric oxide synthase. MPO may also directly catalyse the elimination of nitric oxide. Furthermore, MPO may also increase oxidative stress and oxidise [5, 6] or carbamylate [7] lipoproteins. In these ways, myeloperoxidase activity may directly and indirectly antagonise endothelial function.

Myeloperoxidase is normally expressed in leukocytes, primarily in neutrophils, from which it can be released into the blood stream [8]. Its function in leukocytes is primarily as an antibacterial agent, producing hypochlorous acid to reduce bacterial activity. In humans, myeloperoxidase from the blood stream has been shown to be taken up by endothelial cells as well as traversing the endothelium to become localised in the arterial wall, probably adsorbed to glycosaminoglycans on the endothelial surface and to fibronectin in

the subendothelial extracellular space [9]. It has been demonstrated that intravascular heparin may release significant amounts of MPO from tissues into the blood [10]. The amount released by heparin is greater in patients with coronary artery disease [10], consistent with an inflammatory change in the artery wall causing an accumulation of MPO there.

Myeloperoxidase activity has been suggested to be associated with risk for human cardiovascular disease. It could therefore be assumed that MPO deficiency (a not very rare condition in humans) might be beneficial by lowering the tendency to develop atherosclerosis, although this has not been studied directly in humans. Mouse models of MPO deficiency exist [11], but data from these animals are conflicting. Eiserich et al. [11] reported that young MPO knockout mice have reduced endothelial dysfunction when challenged with lipopolysaccharide. Brennan et al. [12], on the other hand, found increased atherosclerosis in LDL knockout mice on a high-fat diet in which the bone marrow had been repopulated with $MPO^{-/-}$ cells after irradiation. The reason for the latter unexpected result is not clear but may relate either to species differences in pathogenesis of atherosclerosis or in other ways to the complexity of the model. We therefore decided to examine $MPO^{-/-}$ mice further with respect to endothelial function at older age, to determine whether long-standing lack of MPO may enhance or reduce endothelial function. We chose to study the carotid artery, where endothelium-dependent relaxation is largely due to NO, and the saphenous artery, a smaller peripheral vessel, where endothelium-dependent relaxation is partly dependent also on EDHF.

2. Methods

All experiments were approved by the Gothenburg Ethical Committee on Animal Research. Female $MPO^{-/-}$ mice on a C57BL/6 background (generously provided by Dr. S. Baldus, University of Düsseldorf, Germany) and wild-type C57BL/6 control mice aged approximately 8 months were used. A total of 12 knockout and 12 control animals were maintained on standard rat chow (R3, Lantmännen, Sweden). Flow cytometry was used to verify the absence of MPO expression in neutrophils of MPO knockout mice. Seven animals from each group were injected with lipopolysaccharide (LPS) intraperitoneally, 2 mg/kg, 18 hours before the experiment.

2.1. Vascular Preparation. Animals (either untreated or LPS-treated) were euthanized and the right common carotid artery and the right saphenous artery were isolated and rapidly transferred to cold physiological salt solution (PSS; see composition below). Vessels were carefully cleaned from surrounding tissue. The middle part of the carotid artery was divided into two equal parts, as was the middle part of the saphenous artery. The artery segments were transferred to a Mulvany-Halpern type wire myograph (model 600A, Danish Myo Technology, Aarhus, Denmark).

After mounting in the myograph, vessels were equilibrated at 37°C for 30 min. Then the normalization procedure according to Mulvany and Halpern [13] was performed, in which the vessels' circumferences were adjusted to where they can be assumed to develop maximal tension, after which they were equilibrated for another 10–15 min.

A sample of each experiment is given in Figure 3 to illustrate the experimental protocols used. Vessels were then activated 4 times, each for 2 minutes with 5 min intervals between activations (once in high-potassium solution alone, once in phenylephrine (PE) 3 μM alone, once in PE 3 μM in high-potassium solution, and finally again in high-potassium solution alone). The response to the contractions in PE 3 μM in high-potassium solution was taken as the maximal contractile response.

Vessels were then subjected to one of two protocols: one to determine the total response to acetylcholine (Ach) and one where the NO- and prostaglandin-dependent parts of this response were inhibited; the remaining response was defined as EDH-like (similar to what would be expected from endothelium-dependent hyperpolarisation). Thus, one vessel of each type (carotid or saphenous) from each animal was activated with 1 μM noradrenaline (NA) and when contraction approached a plateau, increasing concentrations of Ach were added, each time increasing the Ach concentration a half-log unit over the range 1 nM–10 μM. At the end of the concentration-response experiment, and in the continued presence of 10 μM Ach, 300 μM L-NAME was added. After 20 min, 5 μM indomethacin was added and after another 20 min, 10 μM nitroprusside was added. When relaxation to nitroprusside had reached its maximum, the bath solution was replaced by calcium-free solution without agonists, but still with 10 μM nitroprusside, to determine maximal relaxation.

In the same experiment another part of the vessel of each type from the same animal was activated in parallel following a similar protocol, although L-NAME and indomethacin were added before the concentration-response determination to Ach, in order to determine the EDH-like part of the response to Ach.

Responses were expressed in percent contraction where 100 percent was set to the maximal precontraction level in response to 1 μM phenylephrine.

Vascular responses were recorded as active force (F) and in further analysis were estimated as active tension ($T = F/2l$, where l is length of the vessel segment).

2.2. Preparation of Peripheral Blood Leukocytes (PBL) and Flow Cytometry. One hundred μL of blood was drawn from the tail veins of anaesthetized C57BL/6 and $MPO^{-/-}$ mice. Blood was anticoagulated with Li-heparin and erythrocytes were removed by hypotonic shock using a 155 mM ammonium chloride buffer. Remaining leukocytes were stained with rat anti-mouse Gr1 PE-Cy7 (Pharmingen, San Diego, CA, USA) and mouse anti-mouse MPO FITC (Nordic Biosite, Täby, Sweden). To block unspecific binding of the antibodies, an unlabeled rat anti-mouse FcγII/III antibody (Pharmingen) was added prior to the specific antibodies. Relevant fluorescent isotypic control antibodies, rat IgG2b PY-Cy7 (Pharmingen) and mouse IgG1 FITC (Pharmingen), were used in separate PBL samples to monitor unspecific

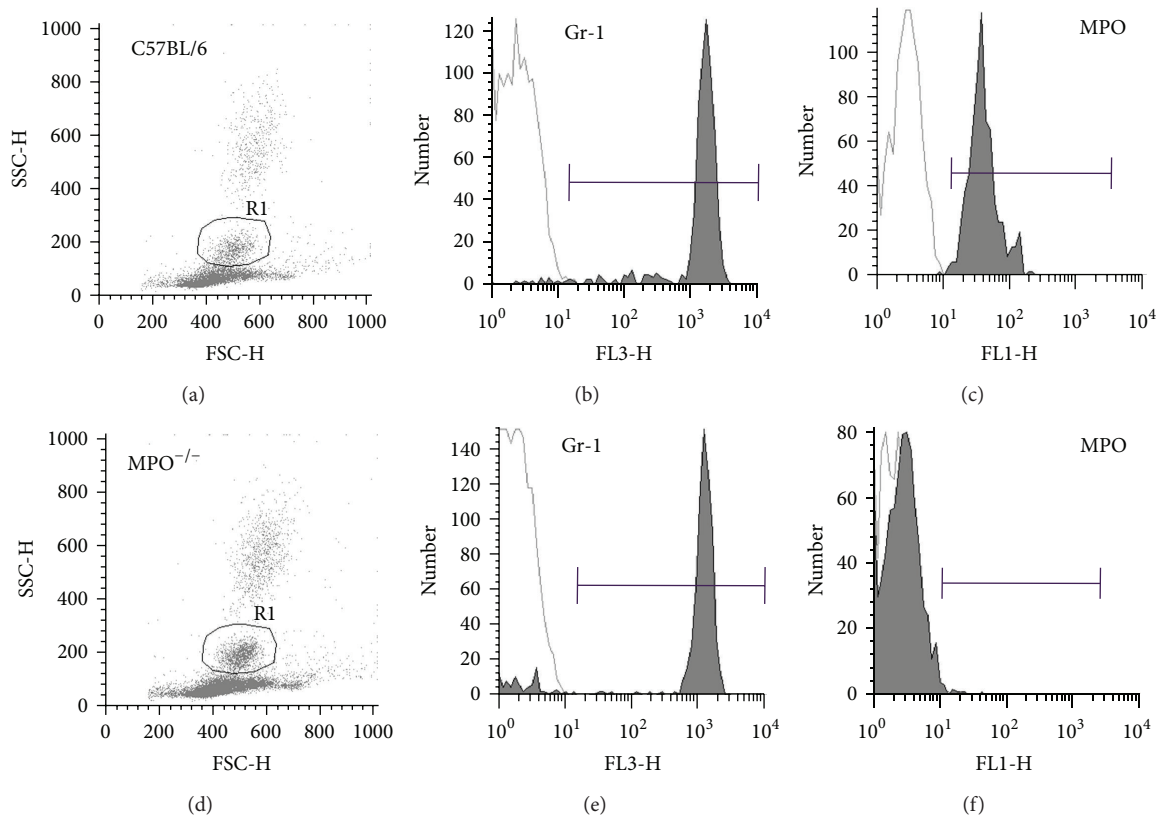

Figure 1: Neutrophils of MPO$^{-/-}$ mice lack MPO expression. Representative flow cytometry data of PBL of C57BL/6 mice (a–c) and MPO$^{-/-}$ mice (d–f). (a and d) Neutrophil gate in forward versus side scatter dot plots of C57BL/6 (a) and MPO$^{-/-}$ mice (d). (b and e) Neutrophil expression of Gr-1 (filled peaks) as compared with isotype controls (open peaks). (c and f) Neutrophil expression of MPO (filled peaks) as compared with isotype controls (open peaks).

binding. Flow cytometry was performed using a FACSCalibur cytometer with an argon laser emitting light at 488 nm (Becton Dickinson, Stockholm, Sweden). From each sample, 5000 cells were acquired and stored in ListMode data files using CellQuest software (Becton Dickinson). The data were analysed using WinList software (Becton Dickinson) and are expressed as mean ± SD percentage of positive cells (above isotypic control) of three mice per group.

2.3. Solutions. The composition of the physiological salt solution (PSS) used was (in mM) as follows: NaCl 119; KCl 4.7; KH_2PO_4 1.18; $MgSO_4$ 1.17; $NaHCO_3$ 25; $CaCl_2$ 2.5; glucose 5.5; EDTA 0.026. In calcium-free PSS, $CaCl_2$ was omitted. High-potassium solution was PSS, where NaCl was substituted with KCl on an equimolar basis. Solutions were equilibrated with 5% CO_2 in O_2 and maintained at 37°C. Drugs were obtained from Sigma.

2.4. Statistics. Statistics was by means of unpaired Student's *t*-test, where two groups were compared, and analysis of variance when comparing a greater number of groups, using GraphPad Prism 4.02 (GraphPad Software, La Jolla, CA, USA) for calculations. Concentration-response relations were analysed by nonlinear fitting to a sigmoidal dose-response relation with variable slope. Inference was based on EC_{50} values and Hill slope. Values are given as means ± SEM.

3. Results

We used flow cytometry to assess MPO expression by peripheral blood neutrophils. As shown in Figure 1, gated neutrophils expressed high level of Gr-1 in both C57BL/6 and MPO$^{-/-}$ mice (87.3 ± 8.3% and 94.2 ± 3.3%, in C57BL/6 and MPO$^{-/-}$ mice, resp.) confirming that the gate indeed selected the neutrophil population. Applying the same gate, most of the C57BL/6 mice neutrophils expressed MPO (96.1 ± 2.5%) while neutrophils of MPO$^{-/-}$ mice indeed were deficient for MPO (0.52 ± 0.51%).

Immunohistochemistry (Figure 2) showed only occasional, faint staining of the endothelium in some control arteries. In distal saphenous arteries, smooth muscle cell nuclei showed slight staining in controls. No straining was seen in MPO$^{-/-}$.

Table 1 presents body weights for the animals as well as normalized diameters for the vessels studied. No significant differences in body weights were found, and also dimensions of carotid arteries were similar between the groups. However,

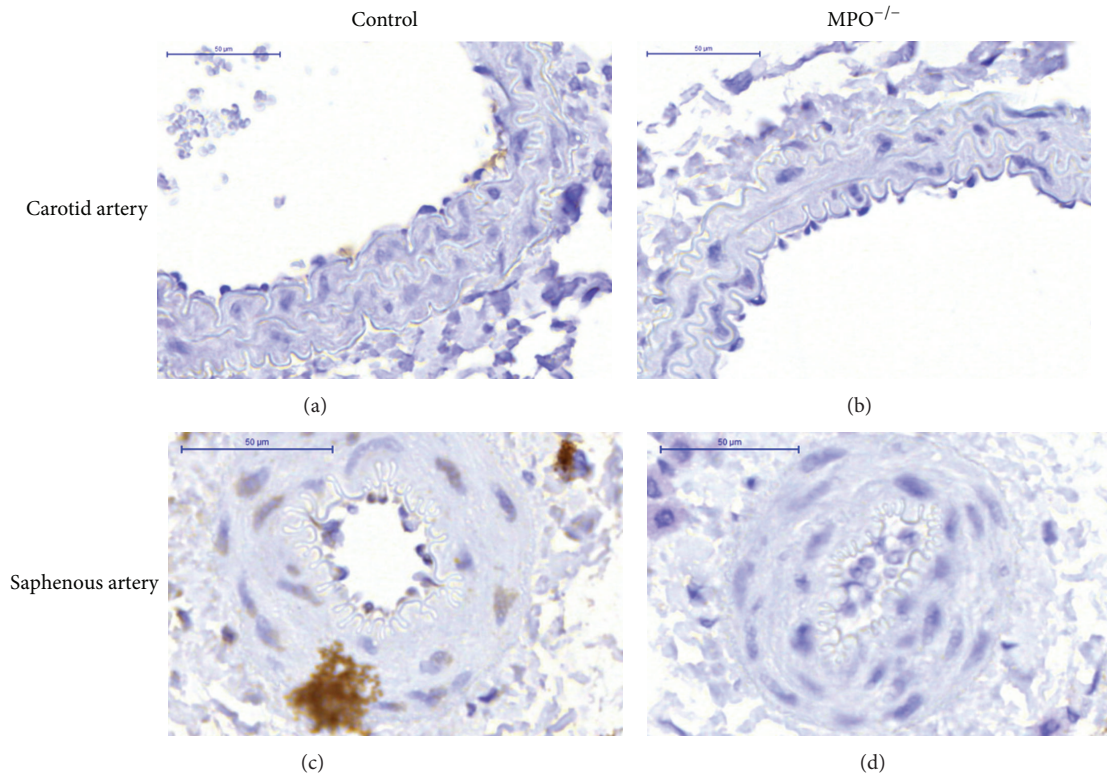

Figure 2: Immunohistochemical detection of myeloperoxidase in carotid (a and b) and saphenous (c and d), arteries from control (a and c) and from MPO$^{-/-}$ (b and d) animals. Myeloperoxidase is brown-stained, seen occasionally in the endothelium and a slight staining in smooth muscle cell nuclei of distal saphenous artery in controls. Scale bar 50 μm.

Table 1: Mouse body weight and diameter of carotid and saphenous arteries in experimental groups.

	MPO$^{-/-}$		Wild-type	
	Control	LPS-treated	Control	LPS-treated
	M ± SEM	M ± SEM	M ± SEM	M ± SEM
Body weight, g	25.80 ± 1.10 $n = 5$	22.88 ± 1.21 $n = 7$	25.05 ± 0.83 $n = 5$	22.72 ± 0.35 $n = 7$
d_{100} carotid, μm	518.79 ± 13.13 $n = 10$	514.09 ± 4.98 $n = 10$	518.70 ± 8.47 $n = 8$	529.62 ± 18.15 $n = 10$
d_{100} saphenous, μm	223.49 ± 6.03** $n = 10$		254.34 ± 7.00 $n = 10$	

** $P < 0.01$.

saphenous arteries were about 10% smaller in MPO$^{-/-}$ animals ($P < 0.01$).

4. Carotid Arteries

No significant differences were found in the responses of carotid arteries to phenylephrine (1 or 3 μM) or potassium either alone or in combination with 3 μM PE (Table 2).

Arteries were activated with 1 μM PE and subsequently acetylcholine was added cumulatively. Increasing concentrations of Ach caused concentration-dependent vasodilatation, but in response to concentrations of 1 μM and higher also transient contractions (Figure 3(a) upper panel). These contractions were not seen in the presence of indomethacin (data not shown). The relaxing response to applied Ach was not significantly different in MPO$^{-/-}$ and wild-type carotid arteries (Figure 4(a), $P = 0.11$).

In the continued presence of the highest concentration of Ach (10 μM), L-NAME and indomethacin were added. This caused a rapid contraction in carotid arteries, which clearly exceeded that evoked by 1 μM PE in the absence of L-NAME. All contractions could be reversed by addition of sodium nitroprusside in both groups (Figure 3(a) upper panel).

If the combination of L-NAME and indomethacin was added before the concentration-response curve, Ach did not cause any dilatation in carotid arteries (Figure 3(b) upper panel).

In comparison to vessels from nontreated animals, vessels taken from LPS-treated animals tended to relax less in response to Ach; this effect of LPS was most pronounced

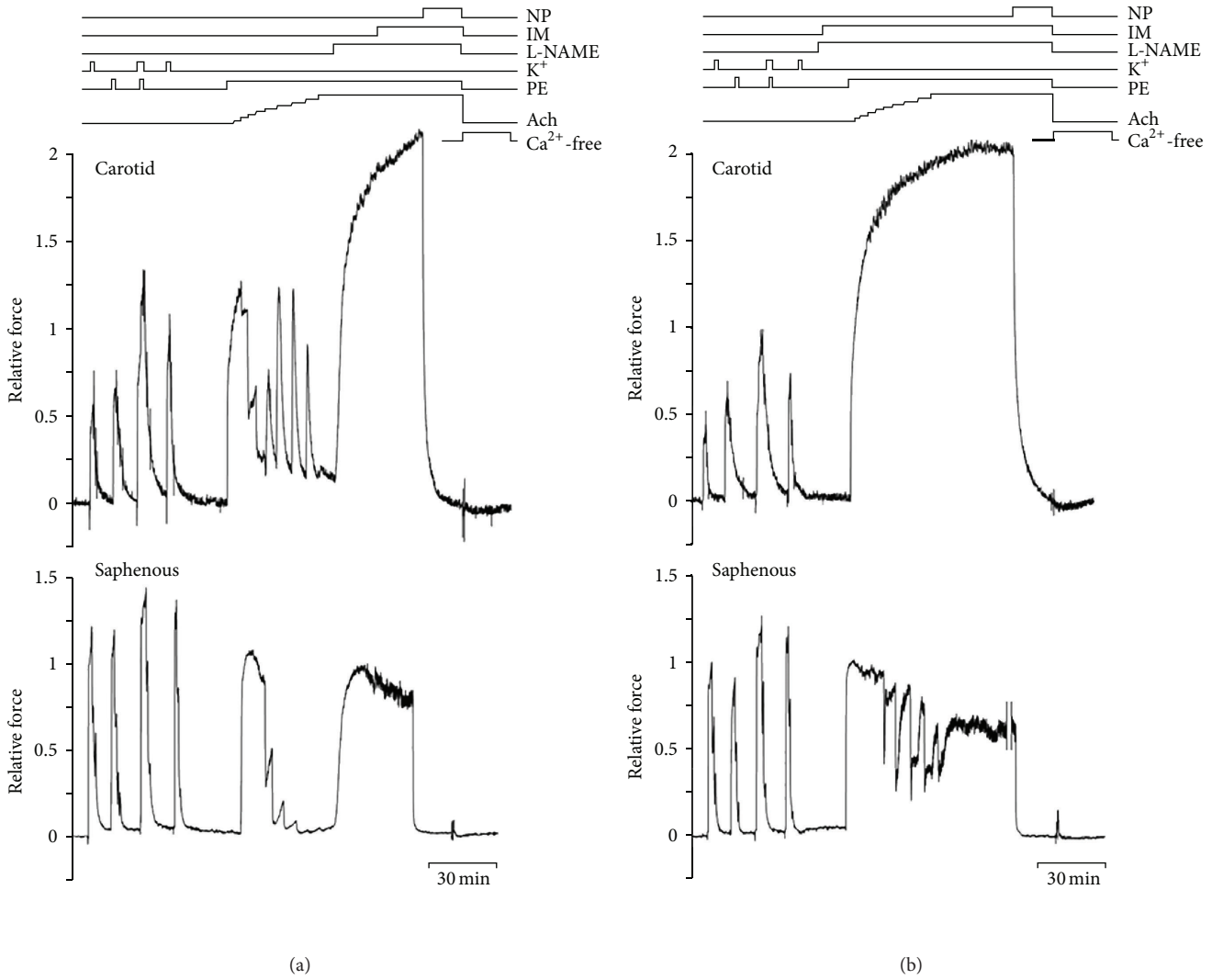

Figure 3: Individual experiments showing the two types of artery and the two protocols used. Original traces from carotid (upper) and saphenous (lower) arteries. In protocol in (a), a concentration-response experiment to acetylcholine was performed before addition of L-NAME and indomethacin; in (b), L-NAME and indomethacin were given before acetylcholine. Note remaining response to acetylcholine in the presence of L-NAME and indomethacin in saphenous arteries (panel (b), lower). NP: nitroprusside, IM: indomethacin, PE: phenylephrine, and Ach: acetylcholine (from 1 nM to 10 μM in half-log steps).

in vessels from MPO$^{-/-}$ animals (Figure 4). Thus, after LPS treatment, carotid arteries from MPO$^{-/-}$ animals showed a significantly smaller response to Ach than arteries from controls (Figure 4(b), $P < 0.01$).

The contraction in response to higher concentration of Ach seen in the carotid arteries was similar in the two groups of animals, whether or not these had been treated with LPS (Figure 5).

5. Saphenous Arteries

Table 3 shows responses to PE and high potassium of saphenous arteries. Responses to high potassium either alone or in combination with 3 μM PE were significantly smaller in the MPO$^{-/-}$ group ($P < 0.01$). Responses to PE were not different with the exception of the response to 1 μM PE in the presence of L-NAME, which also was reduced in MPO$^{-/-}$ ($P < 0.01$).

Saphenous arteries responded with only dilatation to all concentrations of added Ach (Figure 3(a), lower panel). As in the carotid arteries, application of L-NAME and indomethacin in the continued presence of PE and Ach caused maintained contraction. In these arteries, the contraction after application of L-NAME did not exceed that to 1 μM PE alone, in contrast to the carotid arteries. Also in the saphenous arteries contractions were reversed by addition of sodium nitroprusside.

If the combination of L-NAME and indomethacin was added before the concentration-response curve, a dilator response to Ach remained, although of lower amplitude and more transient than in the absence of the inhibitors (Figure 3(b), lower panel). This response was considered to be

Table 2: General data for carotid arteries in experimental groups.

Carotid arteries	$MPO^{-/-}$		Wild-type	
	Control	LPS-treated	Control	LPS-treated
	M ± SEM mN/mm	M ± SEM mN/mm	M ± SEM mN/mm	M ± SEM mN/mm
Contraction (tension)				
1 μM PE	1.31 ± 0.21 $n = 5$	1.46 ± 0.08 $n = 8$	1.51 ± 0.06 $n = 4$	1.52 ± 0.09 $n = 8$
1 μM PE after L-NAME	1.43 ± 0.10 $n = 5$	1.37 ± 0.04 $n = 2$	1.56 ± 0.13 $n = 4$	1.64 ± 0.15 $n = 2$
3 μM PE	0.90 ± 0.11 $n = 10$	1.13 ± 0.06 $n = 10$	1.03 ± 0.07 $n = 8$	1.21 ± 0.06 $n = 10$
3 μM PE in high K^+	1.44 ± 0.11 $n = 10$	1.56 ± 0.06 $n = 10$	1.54 ± 0.11 $n = 8$	1.72 ± 0.11 $n = 10$
High K^+	1.22 ± 0.13 $n = 10$	1.41 ± 0.09 $n = 10$	1.27 ± 0.11 $n = 8$	1.40 ± 0.09 $n = 10$
Relaxation (% of change in tension)				
Maximal relaxation to acetylcholine	92.20 ± 1.15 $n = 5$	93.86 ± 3.88 $n = 8$	94.24 ± 1.36 $n = 4$	94.90 ± 1.65 $n = 8$

Table 3: General data for saphenous arteries in experimental groups.

Saphenous arteries	$MPO^{-/-}$	Wild-type
	Control	Control
	M ± SEM mN/mm	M ± SEM mN/mm
Contraction (tension)		
1 μM PE	2.69 ± 0.41 $n = 5$	2.68 ± 0.40 $n = 5$
1 μM PE after L-NAME	2.91 ± 0.15** $n = 5$	3.59 ± 0.11 $n = 5$
3 μM PE	2.90 ± 0.20 $n = 10$	3.12 ± 0.17 $n = 10$
3 μM PE in high K^+	3.61 ± 0.21** $n = 10$	4.36 ± 0.09 $n = 10$
High K^+	3.36 ± 0.21** $n = 10$	4.03 ± 0.08 $n = 10$
Relaxation (% of change in tension)		
Maximal relaxation to acetylcholine	99.07 ± 0.81 $n = 5$	99.89 ± 0.12 $n = 5$
Maximal relaxation to acetylcholine after L-NAME	94.37 ± 1.60 $n = 5$	97.08 ± 0.50 $n = 5$

** $P < 0.01$.

due to EDHF. Thus, in contrast to the carotid arteries, the Ach response of the saphenous arteries appeared to consist of both L-NAME-inhibited and L-NAME-resistant components.

The concentration-response relation to Ach (in the absence of L-NAME and indomethacin) was right-shifted in vessels from $MPO^{-/-}$ mice in comparison to control vessels (Figure 6(a)). The same was observed after inhibition with L-NAME and indomethacin (Figure 6(b)).

Saphenous arteries from LPS-treated animals were too constricted to permit successful mounting in most cases; therefore, no data are given here.

6. Discussion

This study was motivated by recent interest in the role of myeloperoxidase in the development of endothelial dysfunction. The hypothesis is that the inflammatory response to accumulation and oxidation of lipids in the vascular wall will attract neutrophils, which secrete myeloperoxidase. Liberated MPO may penetrate the endothelium and accumulate extracellularly in the subintimal space, where the activity of MPO will reduce the half-life of nitric oxide, released from the endothelium, and thus counteract NO-dependent vasodilator mechanisms and contribute to endothelial dysfunction. Alternatively products from MPO activity could cause long-lasting damage to the endothelium after initial, transient contacts between neutrophils and endothelial cells.

Toward this background, it was hypothesized that mature animals lacking MPO would show greater NO-dependent relaxation than their normal counterparts, since in the latter group the action of NO would constantly be antagonised by the action of MPO. It was also hypothesized that the $MPO^{-/-}$ animals would be less susceptible to endothelial dysfunction if challenged by LPS. Neither hypothesis could be confirmed in the current study. Thus in carotid arteries, where the Ach response seemed completely dependent on NO (fully blocked by L-NAME), responses to Ach were similar in $MPO^{-/-}$ and controls.

In $MPO^{-/-}$, the saphenous arteries were smaller and had reduced contractile responses to potassium. The latter observation could be secondary to their smaller size, but the reduction in force is proportionately greater than the reduction in size, suggesting also other reasons for the loss of force production. Whether this is related to the lack of MPO remains

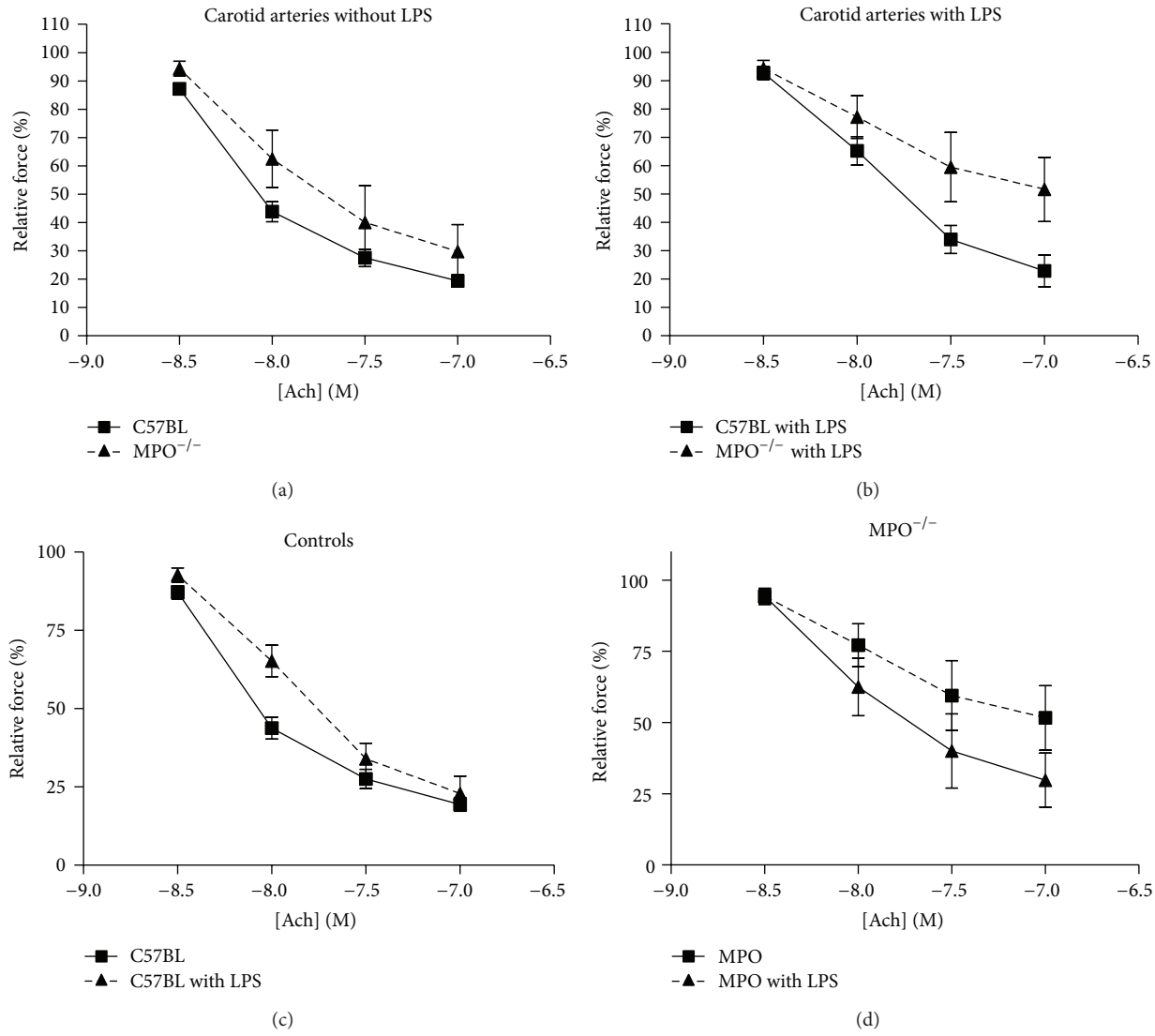

Figure 4: Concentration-response relation for Ach in carotid arteries from untreated animals (a) and from animals treated with lipopolysaccharide (b). Data replotted in (c) for controls and in (d) for $MPO^{-/-}$. Response at maximal relaxation in (b) controls 21 ± 6%, $MPO^{-/-}$ 50 ± 13% ($P < 0.01$).

to be determined. In the saphenous arteries from controls, L-NAME enhanced the response to phenylephrine in contrast to saphenous arteries from $MPO^{-/-}$ and to carotid arteries of either type. This indicates a lesser NO influence and increased endothelial dysfunction in saphenous arteries from $MPO^{-/-}$, which parallels the results of Brennan et al. [12] of increased tendency for atherosclerosis. After NO synthesis inhibition with L-NAME and cyclooxygenase inhibition with indomethacin, the relaxation to Ach was reduced in $MPO^{-/-}$ compared to controls, which suggest an altered EDHF response as well in the knockout animals. It is possible that the EDHF deficiency may in some way relate to the abnormality in the potassium response. Since this endothelial dysfunction was found only in saphenous and not carotid arteries, which differ in respect to the EDHF component, it is possible that the influence of MPO deficiency on endothelial function may primarily relate to the EDHF response.

Eiserich et al. [11] studied isolated aorta from younger mice before and after treatment with a higher dose of LPS for shorter time (12.5 mg/kg for 4 h). They reported very similar concentration-response relations in untreated animals. After LPS treatment, no change in the Ach sensitivity in $MPO^{-/-}$ mice was observed, but a reduced sensitivity in wild-type animals. That observation would be consistent with MPO released from neutrophils during the inflammatory reaction to LPS and accumulating in the vessel wall, reducing the effect of NO liberated from the endothelium. However, our current data do not support such a scheme. Indeed, in the present experiments, carotid arteries from $MPO^{-/-}$ were more vulnerable to LPS treatment than controls, insofar as relaxation to Ach was impaired in these vessels and was little affected in controls. Whether this can be attributed to a different time course of the inflammatory process in the endothelium of these vessels is not known. Although the present study used

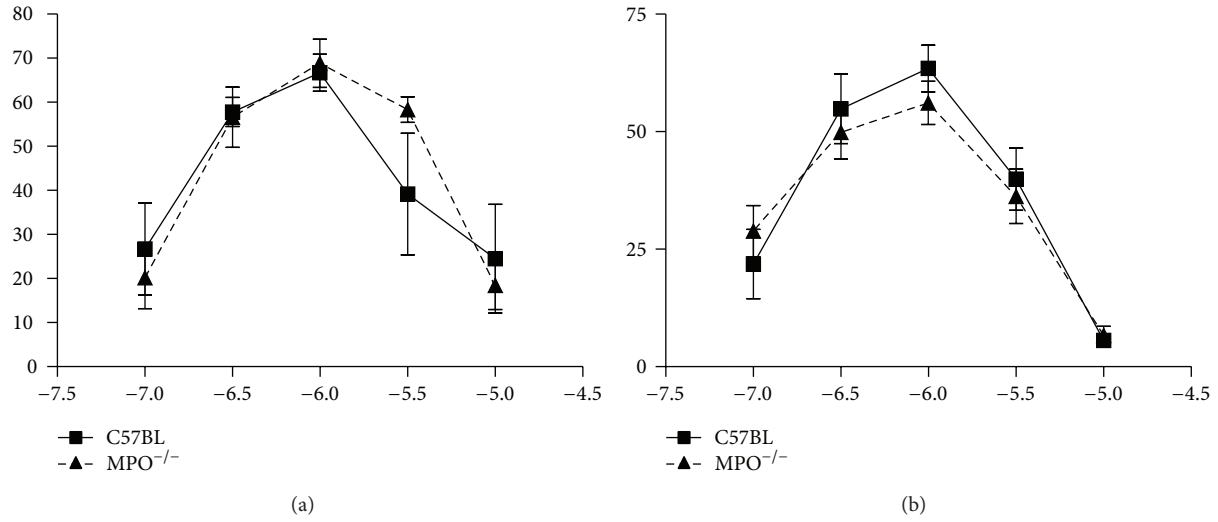

Figure 5: The contractile response to higher concentrations of Ach of carotid arteries from untreated (a) and LPS-treated (b) animals. No differences were seen.

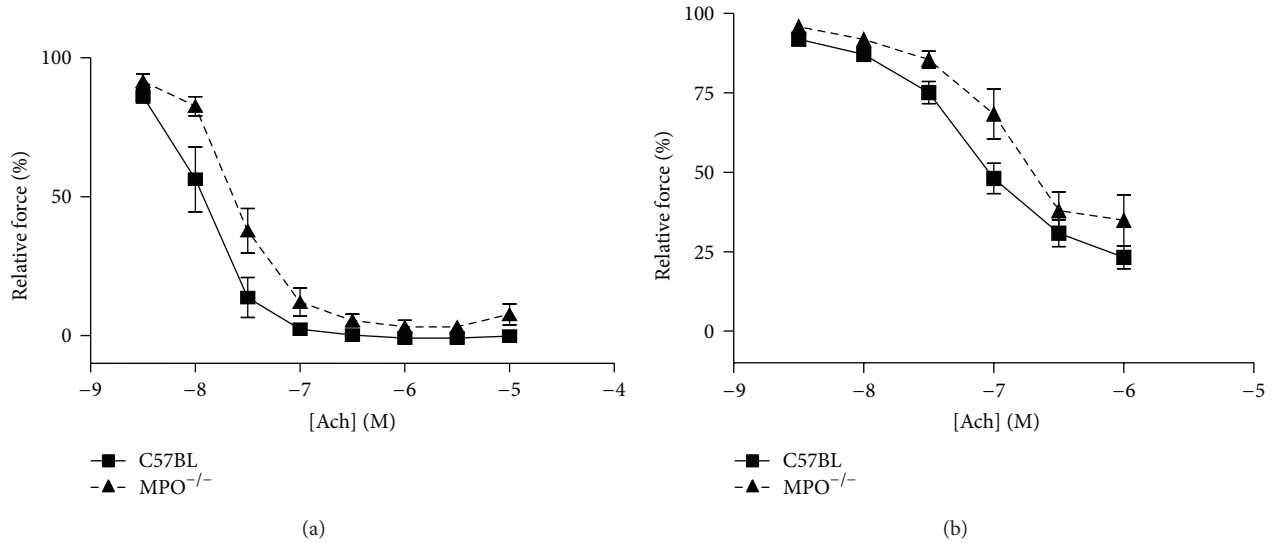

Figure 6: Concentration-response relation for Ach in saphenous arteries in the absence (a) and presence (b) of L-NAME and indomethacin (showing the EDHF response). Vessels from $MPO^{-/-}$ responded less than vessels from wild-type: EC_{50} was in (a) control -7.95 ± 0.045, $MPO^{-/-}$ -7.65 ± 0.041 ($P < 0.001$) and in (b) control -7.19 ± 0.100, $MPO^{-/-}$ -6.98 ± 0.16 ($P < 0.001$).

a different protocol for LPS treatment, this has previously been used successfully to reduce endothelial function in mice [14]. The differences between the studies can obviously also be attributed to gender and age differences. Further studies will be needed to clarify this point.

In response to high concentrations of acetylcholine, contractions rather than relaxations were obtained. These likely correspond to the endothelium-dependent contractile factor (EDCF) that has been demonstrated in other blood vessels [15], where it has been shown to be secondary to production of prostanoids, in particular thromboxane [16]. Constituent with this, the contractions were inhibited by indomethacin in the present experiments. Their nature was not further analysed, but also the EDCF component of the Ach response was unaltered by MPO knockout.

To verify the knockout of the MPO gene product, we used FACS analysis to show elimination of MPO from neutrophilic leukocytes, with unchanged levels of peroxidase activity in eosinophils. This verifies the specificity for neutrophils of MPO in white blood cells. Immunohistochemistry showed faint staining for MPO in the vessel walls from control animals, although transient contacts between white blood cells and endothelium are difficult to demonstrate with this technique.

7. Conclusion

The present study indicates that endothelial function in aged $MPO^{-/-}$ mice is not significantly different from control. If

MPO plays a significant role in degradation of NO, one might expect this system to be affected by life-long enhancement of NO effects. This does not seem to be the case here, although we cannot exclude that altered sensitivity to NO and an altered EDHF response are adaptive changes in response to altered NO levels.

Disclosure

This work was performed in-house at AstraZeneca R&D Mölndal, Sweden, where three of the authors (Ulla Brandt-Eliasson, Li-Ming Gan, and Martin Kjerrulf) are employed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] A. Wennmalm, "Endothelial nitric oxide and cardiovascular disease," *Journal of Internal Medicine*, vol. 235, no. 4, pp. 317–327, 1994.

[2] M. Félétou and P. M. Vanhoutte, "Endothelial dysfunction: a multifaceted disorder," *The American Journal of Physiology*, vol. 291, no. 3, pp. H985–H1002, 2006.

[3] T. J. Anderson, M. D. Gerhard, I. T. Meredith et al., "Systemic nature of endothelial dysfunction in atherosclerosis," *The American Journal of Cardiology*, vol. 75, no. 6, pp. 71B–74B, 1995.

[4] M.-L. Brennan and S. L. Hazen, "Emerging role of myeloperoxidase and oxidant stress markers in cardiovascular risk assessment," *Current Opinion in Lipidology*, vol. 14, no. 4, pp. 353–359, 2003.

[5] C. Bergt, S. Pennathur, X. Fu et al., "The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 35, pp. 13032–13037, 2004.

[6] L. Zheng, B. Nukuna, M.-L. Brennan et al., "Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and function impairment in subjects with cardiovascular disease," *Journal of Clinical Investigation*, vol. 114, no. 4, pp. 529–541, 2004.

[7] S. Sirpal, "Myeloperoxidase-mediated lipoprotein carbamylation as a mechanistic pathway for atherosclerotic vascular disease," *Clinical Science*, vol. 116, no. 9, pp. 681–695, 2009.

[8] J. Arnhold, "Properties, functions, and secretion of human myeloperoxidase," *Biochemistry*, vol. 69, no. 1, pp. 4–9, 2004.

[9] S. Baldus, J. P. Eiserich, A. Mani et al., "Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration," *Journal of Clinical Investigation*, vol. 108, no. 12, pp. 1759–1770, 2001.

[10] S. Baldus, V. Rudolph, M. Roiss et al., "Heparins increase endothelial nitric oxide bioavailability by liberating vessel-immobilized myeloperoxidase," *Circulation*, vol. 113, no. 15, pp. 1871–1878, 2006.

[11] J. P. Eiserich, S. Baldus, M.-L. Brennan et al., "Myeloperoxidase, a leukocyte-derived vascular NO oxidase," *Science*, vol. 296, no. 5577, pp. 2391–2394, 2002.

[12] M.-L. Brennan, M. M. Anderson, D. M. Shih et al., "Increased atherosclerosis in myeloperoxidase-deficient mice," *Journal of Clinical Investigation*, vol. 107, no. 4, pp. 419–430, 2001.

[13] M. J. Mulvany and W. Halpern, "Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats," *Circulation Research*, vol. 41, no. 1, pp. 19–26, 1977.

[14] J. Wikström, *Imaging of coronary artery function and morphology in living mice [Doctoral Thesis]*, 2007.

[15] R. F. Furchgott and P. M. Vanhoutte, "Endothelium-derived relaxing and contracting factors," *The FASEB Journal*, vol. 3, no. 9, pp. 2007–2018, 1989.

[16] E. H. C. Tang, D. D. Ku, G. L. Tipoe, M. Feletou, R. Y. K. Man, and P. M. Vanhoutte, "Endothelium-dependent contractions occur in the aorta of wild-type and COX2-/- knockout but not COX1-/- knockout mice," *Journal of Cardiovascular Pharmacology*, vol. 46, no. 6, pp. 761–765, 2005.