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Oxidative Stress, Vascular Remodeling, and Vascular Inflammation in Hypertension

Guest Editors: Nicolas Federico Renna, Roberto Miguel Miatello, and Natalia de las Heras

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Editorial Board

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Editorial

Oxidative Stress, Vascular Remodeling, and Vascular Inflammation in Hypertension

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Hypertension is a major public health problem worldwide, affecting over 50 million individuals in the United States alone. The modern history of hypertension begins with the understanding of the cardiovascular system with the work of the physician William Harvey (1578–1657), who described the circulation of blood in his book *De Motu Cordis*. The English clergyman Stephen Hales made the first published measurement of blood pressure in 1733. Frederick Akbar Mahomed (1849–1884) made the first report of elevated blood pressure in a person without evidence of kidney disease. However, hypertension as a clinical entity came into being in 1896 with the invention of the cuff-based sphygmomanometer by Scipione Riva-Rocci. This apparatus has allowed the measurement of the blood pressure into the clinic. In 1905, Nikolai Korotkoff improved the technique by describing the Korotkoff sounds that are heard when the artery is auscultated with a stethoscope while the sphygmomanometer cuff is deflated.

The concept of essential hypertension ("hypertonic essential") was introduced in 1925 by the physiologist Otto Frank to describe elevated blood pressure for which no cause could be found. Over the several decades, increasing evidence accumulated from actuarial reports and longitudinal studies, such as the Framingham Heart Study. In the actuality, the term "inflammation" is used in the context of cardiovascular disease as a catchall referring to nonspecific phenomena, such as elevation of C-reactive protein. Most clinicians and investigators find this vague and difficult to understand. In this issue, we will attempt to address some of these puzzling questions.

In this way, several authors write on oxidative stress in the pathophysiology of target organ damage. G. Csányi and P. J. Pagano postulated that peptides mimicking the Nox4 B-loop and regions C-terminus on inhibit Nox4 activity and their findings suggest that Nox4 exists in a tightly assembled and active conformation which, unlike other Noxes, cannot be disrupted by conventional means. R. O. Marañon et al. demonstrated, in nonischemic 5/6 nephrectomized rat (NefR), a model of chronic kidney disease, that tempol improves NO-contents and basal tone, without decrease MAP, indicating that oxidative stress could be implicated early and independently to hypertension, in the vascular alterations. B. P. Campagnaro et al. proposed that the non-hemodynamic effects of angiotensin II are important for the damage observed in the two-kidney, one-clip (2K1C) renovascular hypertension model and demonstrated that 2K1C hypertensive mice have an elevated lymphocyte count, while undifferentiated bone marrow mononuclear cells counts were diminished.

N. Arias et al. describe mechanisms associated with inflammation and oxidative stress in the brain. N. Arias et al., in their study, examined the differential contribution of these leading factors to the oxidative metabolism of diverse brain limbic system regions frequently involved in memory process by histochemical labelling of cytochrome oxidase (COx). S.-L. Chan and G. L. Baumbach examined the role of genetic deficiency of NAD(P)H-oxidase subunit Nox2 in the function

and structure of cerebral arterioles during hypertension and concluded that hypertension-induced superoxide production derived from Nox2-containing NADPH oxidase promotes hypertrophy and causes endothelial dysfunction in cerebral arterioles, possibly involving interaction with nitric oxide.

On the other hand, J. Alvarez-Camacho et al. describe the pathophysiologic link between the metabolic syndrome, obesity, hypertension, and adipokines at experimental and clinical level. J. Alvarez-Camacho et al. addresse topics related to modern aspects in the pathophysiology of hypertension such as the role of inflammation, environment, epigenetics, and adiponectin in hypertension, and Y. Mendizabal et al. discuss the central role of hypertension and vascular inflammation in metabolic syndrome and the role of sympathetic tone. Subsequently, they examine the link between endothelial dysfunction and insulin resistance. Finally, animal models used in the study of vascular function of metabolic syndrome are reviewed, in particular, the Zucker fatty rat and the spontaneously hypertensive obese rat (SHROB).

N. F. Renna et al. conducted a review of the mechanisms involved in the physiopathological vascular changes, adaptive and maladaptive, called vascular remodeling and the involvement of inflammatory mechanisms.

Finally, D. M. Babbitt et al. performed their analysis on a clinical protocol that demonstrated that aerobic exercise training might be a viable, nonpharmacological method to improve inflammation status and endothelial function and thereby contribute to risk reduction for cardiovascular disease in African Americans.

In conclusion, the observed elevation in inflammatory parameters in subjects who subsequently go on to develop hypertension is particularly relevant and creates options for potential primary prevention strategies. A number of therapeutic agents have been identified which are able to influence this inflammatory process and positively influence cardiovascular outcomes.

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Review Article

Pathophysiology of Vascular Remodeling in Hypertension

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Vascular remodeling refers to alterations in the structure of resistance vessels contributing to elevated systemic vascular resistance in hypertension. We start with some historical aspects, underscoring the importance of Glagov's contribution. We then move to some basic concepts on the biomechanics of blood vessels and explain the definitions proposed by Mulvany for specific forms of remodeling, especially inward eutrophic and inward hypertrophic. The available evidence for the existence of remodeled resistance vessels in hypertension comes next, with relatively more weight given to human, in comparison with animal data. Mechanisms are discussed. The impact of antihypertensive drug treatment on remodeling is described, again with emphasis on human data. Some details are given on the three mechanisms to date which point to remodeling resistance arteries as an independent predictor of cardiovascular risk in hypertensive patients. We terminate by considering the potential role of remodeling in the pathogenesis of endorgan damage and in the perpetuation of hypertension.

1. Introduction

In 1987, Glagov et al. reported the surprising finding that atherosclerotic narrowing of the arterial lumen is not simply the result of the enlargement of atherosclerotic lesions [1]. They described that the arteries, instead of remodeling the narrowed lumen, undergo many changes, such as increasing the outside diameter, to preserve blood flow.

This adaptability of the arteries is essential in arterial diseases. As with atherosclerotic coronary disease, peripheral vascular disease and hypertension may be considered a failure of the arterial wall to maintain a suitable mesh size to allow normal blood flow.

Recently, it has been suggested that the inability to remodel vessels properly is a way of "vascular insufficiency," similar to that observed in the heart during heart failure. A definition of failure must begin with a description of the normal mechanisms that allow the artery walls to adapt to physiological requirements.

Hypertension elicits two different kinds of diffuse structural changes in the systemic microcirculation. One, termed rarefaction, consists in an abnormally low spatial density of arterioles, capillaries, and possibly venules. The other concerns structural modifications of resistance small arteries and arterioles, which lead to a reduction in lumen diameter and are grouped under the generic name of remodeling. We have recently reviewed rarefaction in detail. The focus of the present paper is on remodeling which probably accounts for the major part of long-term elevation of systemic vascular resistance (SVR) in hypertensive patients [2–9].

2. Definition of Vascular Remodeling

The vascular wall is formed by endothelium cells, smooth muscle cells, and fibroblasts interacting to form an autocrine-paracrine complex. During vascularization, the vascular wall detects changes in the environment, integrates these intercellular communication signals, and, through the local production of mediators, influences vascular structure and function. Vascular remodeling is an active process of structural change that involves changes in at least four cellular processes:

cell growth, cell death, cell migration, and the synthesis or degradation of extracellular matrix. Vascular remodeling is dependent on dynamic interactions between local growth factors, vasoactive substances, and hemodynamic stimuli and is an active process that occurs in response to long-standing changes in hemodynamic conditions; however, it may subsequently contribute to the pathophysiology of vascular diseases and circulatory disorders [10].

In the historical work cited previously, the increased Wof hypertensive resistance vessels were uniformly ascribed to a higher volume of wall material per unit length of vessel (increased wall cross-sectional area (CSA)) or "hypertrophy." It was assumed that smooth muscle cells in resistance vessels behaved as did left ventricular myocytes in the face of the increased pressure load and that growth took place mainly on the luminal side, leading to a structural reduction of internal diameter. The term remodeling was first applied to resistance vessels by Baumbach and Heistad, based on observations made in pial arterioles from stroke-prone spontaneously hypertensive rats (SPSHRs), to indicate a structural rearrangement of existing wall material around a smaller lumen [11–13].

Mulvany proposed that vascular remodeling should encompass any change in diameter noted in a fully relaxed vessel, not explained by a change in transmural pressure or compliance, and therefore due to structural factors [14–16].

To be operational, the classification necessitates appropriate methods for the measurement of resistance vessels dimensions. This problem is much harder than would seem at first sight.

To meet the definition of remodeling given previously, the respective sizes of hypertensive and normotensive small arteries and arterioles must be compared with the influence of the following factors either removed or controlled for: (i) vascular tone, (ii) transmural pressure, and (iii) vessel compliance.

Obviously, none of these requirements would be met by geometrical measurements made on standard histological sections prepared without perfusion of the tissue sample (e.g., shrinking artefacts) [17, 18].

One widely used approach, possible with small arteries (100 and 300 mm), is to carry out geometrical measurements on dissected segments put in standardized conditions in vitro.

3. Classification of Vascular Remodeling

Consideration of morphological changes has changed over time. Feihl et al. [2] proposed a classification based on the response to increased blood pressure. These changes are displayed predominantly in media-to-lumen ratio (M/L), changing the vessel wall width for increased muscle mass (Figure 1(A)) or in the reorganization of cellular and noncellular elements (Figure 1(B)). These changes increase vascular reactivity, thus enhancing peripheral resistance. Another mechanism mainly involves changes in the dimensions of the lumen (Figures 1(C) and 1(D)). In this case, the restructuring of the active components and cell signals does not result in significant changes in the dimensions of the vascular lumen;

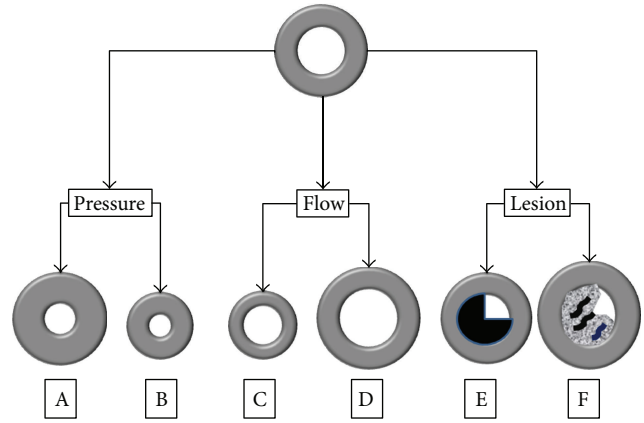

Figure 1: Changes are predominantly in media-to-lumen ratio (M/L), changing the vessel wall width for increased muscle mass (Figure 1(A)) or in the reorganization of cellular and noncellular elements (Figure 1(B)). Another mechanism of remodeling mainly involves changes in the dimensions of the lumen (Figures 1(C) and 1(D)). In this case, the restructuring of the active components and cell signals does not result in significant changes in the dimensions of the vascular lumen. Another form of vascular remodeling is microcirculation rarefaction (Figures 1(E) and 1(F)).

the changes in vessel wall thickness are relatively small. Clinical examples of this type of restructuring include the dilation of vascular remodeling associated with a constantly high blood flow (Figure 1(D)) (e.g., arteriovenous fistula) or the loss of cellularity and extracellular matrix proteolysis, resulting in the formation of an aneurysm. Equally, a reduction in the diameter of the vascular mass results from a long-term reduction in blood flow (Figure 1(C)). In fact, microcirculation rarefaction (loss of the capillary zone) is another form of vascular remodeling that promotes hypertension and ischemic tissue. The architecture of the vascular wall is also markedly changed in response to vascular injury (Figures 1(E) and 1(F)). Neointima forms as part of the reparative response to injury, and its formation involves thrombosis, migration and vascular smooth muscle cells (VSMC) proliferation, matrix production, and the infiltration of inflammatory cells.

Hypertension is associated with structural changes in the resistance vessels such as reduction in lumen diameter and increase in M/L ratio. This mode of structural change has been called "remodeling" [19].

Structural changes in resistance vessels are described as a rearrangement process to understand the pathogenesis of the disease and its therapeutic approach. However, it has been discussed that the term "remodeling" is not ideal because it is frequently used to describe any change in the structure of the vessel or myocardium. To avoid this difficulty, some authors make four proposals [20].

First, the term "remodeling" is limited to situations where there is a change in the lumen of a relaxed vessel, as measured under standard intravascular pressure. The changes in the characteristics of the wall material do not take into account the change in the vascular lumen.

Second, the process of changing the vessel wall without changes in the amount or characteristics of the materials is termed eutrophic remodeling. This process can be characteristic of situations involving an increase in the amount of material (hypertrophic remodeling) and those involving a reduction in the amount of material (hypotrophic remodeling).

Third, changes associated with decrease or increase in lumen diameter should be classified as internal remodeling and external remodeling, respectively.

Finally, the remodeling process should be quantified. The term "remodeling index" refers to the variations of lumen referred to as eutrophic remodeling, depending on the changes in the wall section area.

The previous four proposals allow for accurate terminology. Thus, the increase in the M/L ratio and decrease in the lumen diameter in resistance vessels of patients with essential hypertension without any change in the amount of wall material are called inner eutrophic remodeling. The decrease in the lumen diameter of the renal afferent arteriole with a decrease in the amount of wall material is called inner hypotrophic remodeling.

Chronic changes in hemodynamic forces structurally alter the vascular wall. In addition, hemodynamic changes are not the only production mechanisms of vascular remodeling. The inflammatory response and changes in the components of the matrix have been suggested as important mediators in the vascular adaptation process [21].

Figure 2 highlights schematically the adaptation of these changes in different pathologies, including structural changes to the intima layer that contribute to remodeling of the vascular wall. Thus, outward remodeling compensates for atherosclerotic plaque growth and delays the progression of blood flow limitation during stenosis, whereas during restenosis, intimal hyperplasia causes a narrowing of the lumen.

In summary, vascular wall remodeling is the result of changes in cellular and noncellular components, depending on the disease process causing the changes. Changes in the growth and migration of VSMC, endothelial dysfunction, inflammatory processes, and the synthesis or degradation of extracellular matrix components may be present during the disease process.

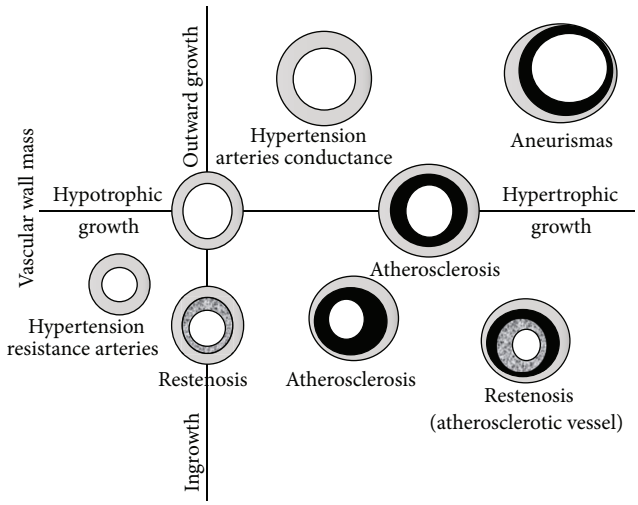

Figure 2: Schematic representation for the adaptation of these changes in different pathologies, including structural changes to the intima layer that contribute to remodeling of the vascular wall. Thus, outward remodeling compensates for atherosclerotic plaque growth and delays the progression of blood flow limitation during stenosis, whereas during restenosis, intimal hyperplasia causes a narrowing of the lumen.

4. Pathophysiology of Vascular Remodeling in Hypertension

4.1. Hypothesis of Inflammatory and Endothelial Dysfunction. The traditional view of atherosclerosis as a lipid storage disease is crumbling with growing evidence that inflammation is involved during all stages, from the initial injury to the final stage of thrombotic complications. The narrowing of the arterial lumen is not necessarily a sign of myocardial infarction, and treating narrowed blood vessels does not prolong life. Although invasive procedures are needed in some cases, we understand that medical treatment and lifestyle modification (diet and physical activity) produce benefits that may result from reductions in inflammatory processes [22].

Usually, endothelial cells (EC) prevent leukocyte adhesion. However, the triggers of atherosclerosis can initiate the expression of adhesion molecules on EC, mediating leukocyte adhesion to the arterial wall. A key part of this interaction is VCAM-1. It is likely that oxidized lipids can induce gene expression via the pathway initiated by the nuclear transcription factor κB (NF-κB), such as IL-1β and TNF-α [23].

This concept of vascular inflammatory disease allows a new approach for risk stratification and treatment. Increased levels of CAM are predictive of cardiac events and are an independent risk factor in men with coronary disease [24]. In our previous study, we demonstrated the presence of the endothelium as well as the products of NF-κB signaling and VCAM-1 in an experimental model of metabolic syndrome in hypertensive rats receiving a fructose-rich diet (FFHR) [25].

Chemokines are low molecular weight cytokines responsible for mediating the maturation, differentiation, and migration of cells involved in the inflammatory response. In addition to this role, chemokines could promote reactive oxygen species (ROS) production and other cytokines during leukocyte infiltration of the vessel wall. Monocyte chemotactic protein-1 (MCP-1) is a chemokine that regulates the migration and infiltration of monocytes and macrophages into the site of inflammation. It is overexpressed in the presence of cardiovascular risk factors, especially in atherosclerotic lesions. Differential activation induces nuclear transcription factors such as NF-κB and AP-1, which leads to the release of IL-6 and the proliferation of VSMC [26].

Cytokines are soluble proteins that form a complex signaling network critical in the regulation of innate and adaptive inflammatory response. Cytokines modulate the inflammatory response through their influence on the growth,

development, and activation of leukocytes and other inflammatory cells. TNF-α is a key mediator in systemic inflammation with a significant role in the Th1 inflammatory pathway. The activity of TNF-α is varied and includes the production of interleukin CAM expression, cell migration and activation, and activation of metalloproteinases (MMP) and COX activity, promoting the procoagulant state. TNF-α is detected in endothelial cells and smooth muscle cells at all stages of the formation of atheromatous plaques [27].

There are over 30 members of the interleukin family. They are subdivided by the similar structure or homology of the receptor. The transformation from a vascular homeostasis inflammatory state is influenced by an imbalance between the proinflammatory and anti-inflammatory activities of interleukins. The role of IL-1 includes the stimulation of CAM, chemokines, growth factors, tissue factor, and other cytokines. The expression levels of the receptor antagonist IL-1Ra significantly increase in unstable angina compared with stable angina. Decreased levels of IL-1Ra after coronary stent placement may be linked to a low association with recurrent ischemia [28]. IL-6 is a multifunctional cytokine with a central role in inflammation. Elevated levels of IL-6 increase the risk of myocardial infarction and mortality in patients with coronary heart disease [29].

IL-10 has pleiotropic properties and influences different cell populations. Its most important role is in inflammatory vascular disease as part of the Th2 response. The expression of IL-10 decreases the expression of inflammatory cytokines, decreasing the Th1 phenotype. IL-10 also decreases NF-κB signaling reducing synthesis of proinflammatory cytokines, CAM, chemoattractants, and growth factors [30, 31].

Endothelial dysfunction in FFHR causes an increase in the expression of NF-κB and AP-1 and the posttranscriptional product VCAM-1. The expression of NF-κB (p65) and AP-1 (c-fos) predominates throughout the vessel wall. Increased VCAM-1, as discussed in the literature, is a marker of vascular inflammation, vascular permeability, and endothelial dysfunction.

This experimental model produced an increased expression of several cytokines. This finding demonstrates that the vascular bed FFHR model presents a proinflammatory and proatherogenic microenvironment that favors vascular remodeling. C-reactive protein (CRP) was used to evaluate whether this local inflammatory process is also systemic and revealed significantly increased IL-6 expression in the liver.

The potential importance of vascular wall inflammation as a therapeutic target remains an area not yet fully explored, where understanding the involvement of inflammatory mediators in vascular remodeling is relevant. The data suggest that oxidative stress and the subsequent activation of genes involved in the inflammatory process are actively involved in organ damage at the vascular level.

4.2. Vascular Remodeling and Extracellular Matrix Metalloproteinases. MMPs are tools for maintaining the homeostasis of extracellular structures. Their synthesis is induced by cytokines as well as cell-cell and cell-matrix interactions. Acute coronary syndromes are an example of an increase in clinical conditions, specifically in the vulnerable region of the plaque [32]. Exposure to oxidized low-density lipoproteins or TNF-α induces the expression of MT3-MMP, a protease that degrades atherosclerotic plaques and is expressed in macrophages [33, 34].

MMPs with accessory signaling molecules can modulate cell-cell interactions through the activation of signal transmission and release of cytokines and chemokines. By these effects, accessory signaling molecules can propagate the inflammatory response.

4.3. Vascular Remodeling and Acute Phase Reactants. The production of acute phase reactants is a normal physiological response to cytokine release in acute and chronic inflammatory conditions. Ultrasensitive quantification of CRP, when it is below the detection limits of the common assay, has a very important role in the detection of vascular inflammation and cardiovascular risk prediction. There is evidence that CRP is involved in atherosclerosis, especially during the early stages. It stimulates the production of proinflammatory cytokines in monocytes and macrophages [35] and mediates the expression of CAM, allowing for increased leukocyte adhesion and migration. Their increased expression suppresses endothelial nitric oxide synthase [36] and promotes a procoagulant state.

Multiple studies have determined that increases in CPR are an independent risk factor for developing atherosclerosis. Data from clinical studies indicate that this association is less important when viewed in healthy subjects and controls inflammatory markers such as IL-6 and fibrinogen [37, 38], whereas another study identified CRP as a predictor of diabetes mellitus independent of established risk factors. CRP also indicated a correlation with the risk of cardiovascular events in women with metabolic syndrome [39].

4.4. Vascular Remodeling and the Renin-Angiotensin-Aldosterone System. Another important pillar in the vascular remodeling process is the RAAS [40, 41]. To evaluate its participation, we studied the expression of AT1R and AT2R at the vascular level. In the experimental model of FFHR, we observed increased expression of AT1R and decreased expression of AT2R, promoting growth, vascular hypertrophy, and endothelial dysfunction. The release of ROS and initiation of vascular inflammation through different intracellular signaling cascades foster interconnections with other routes such as NAD(P)H oxidase and the growth factor receptor associated with insulin (IGFR).

Figure 3 allows us to appreciate the AT1R-associated intracellular cascades. In this experimental model, the route associated with the satellite receptor and the IGFR subunit associated with NAD(P)H oxidase are the most important pathophysiological mechanisms. The FAK pathways PI3K and JAK2 generate stimuli and trigger contraction, migration, and cell adhesion via intranuclear promoters that synthesize ICAM-1 and VCAM-1. EGFR and IGFR amplified pathways are associated with cellular growth and hypertrophy as a result of insulinogenic stimuli and permit activation of collagenase, which modifies the extracellular matrix. Finally,

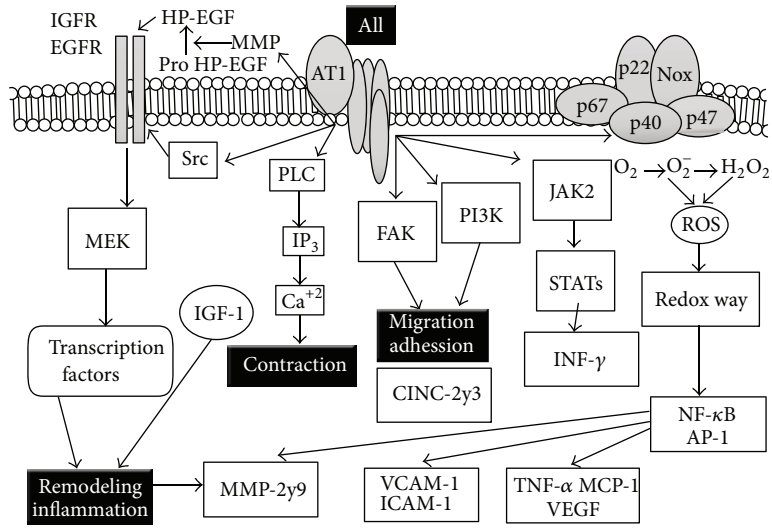

Figure 3: Associated intracellular cascades to physiopathology of vascular remodeling. In FFHR experimental model, the route associated with the satellite receptor and the IGFR subunit associated with NAD(P)H oxidase are the most important pathophysiological mechanisms. Also, the oxidative stress pathway stimulated by angiotensin activates redox-sensitive inflammatory molecules such as AP-1 and NF-κB, which amplify vascular inflammatory response.

the oxidative stress pathway stimulated by angiotensin activates redox-sensitive inflammatory molecules such as AP-1 and NF-κB, which amplify the inflammatory response by cytokines, chemokines, and lymphokines to ultimately induce more vascular inflammation.

Angiotensin II is the main effector of the RAAS in the homeostatic regulation of the cardiovascular system and in the pathogenesis of cardiovascular disease. Aldosterone interacts with mineralocorticoid receptors (MR), causing endothelial dysfunction, facilitating thrombosis, reducing complacence, causing vascular hypertrophy and cardiac fibrosis and generating pathological remodeling. Aldosterone also induces the growth and proliferation of VSMC. A classical genomic action of aldosterone on MR is the translocation of this Aldo-MR complex into the nucleus, where it interacts with promoters to posttranscriptionally regulate gene and protein expression. For this path, increased Ki-ras2A expression (small and monomeric GTP-binding protein), which is associated with cardiac remodeling, generates fibrosis and cell proliferation by ERK1/2 possibly [42]. Recently, some authors have demonstrated that aldosterone stimulates EGFR intracellularly in CHO cells. The transactivation of this receptor has also been described as a crucial step in the cascade of MAPK signaling activated by angiotensin II. This pathway allows for "cross-talk" and mutual activation that allows the development of cardiovascular injury and subsequent remodeling. The latter route is via "fast" activation, which is different from genomic stimulation and stimulates MKP-1 and Ki-generated ras2A proliferation and vascular remodeling; this discovery explains the changes previously observed in other studies [43].

Noting the role of aldosterone in vascular remodeling in FFHR, we observed that chronic administration of spironolactone did not change the variables of metabolic syndrome that were partially reversed by oxidative stress. This can be explained by the relationship between aldosterone and the angiotensin II receptor AT1R, which sensitizes the effects and increased the postreceptor response [41].

In summary, abundant lines of evidence indicate the involvement of the RAAS in the pathophysiology of vascular remodeling; our observations in experimental pathology highlight the structural and functional changes.

In this special issue, different authors have tried to demonstrate the involvement of different pathophysiological mechanisms to clarify the vascular changes associated with hypertension and metabolic syndrome.

5. Clinical Data

The most feasible possibility for quantitative structural studies of resistance vessels in humans relies on the examination of small muscular (presumably resistance) arteries from biopsies of subcutaneous gluteal fat carried out under local anaesthesia. Small arteries can also be obtained from omental fat excised at the time of abdominal surgery [12, 44–47]. The dissected vessels are mounted in a wire or pressure myograph and characterized with the aforementioned methodology. Due to the invasive character of these procedures, most relevant studies are of modest size, typically involving between 10 and 20 subjects per group (with a few notable exceptions 49–51). Furthermore, untreated hypertensives are often patients in whom medication was withdrawn for a few weeks, rather than being newly diagnosed.

In several studies, data indicate that small subcutaneous arteries of nondiabetic hypertensives undergo inward eutrophic remodeling. In contrast, it appears that diabetes on top of essential hypertension is associated with media hypertrophy, without a reduction of lumen diameter as measured in passive conditions. The same hypertrophy was also shown by one of these studies in normotensive diabetics, supporting

a pressure-independent effect of diabetes on resistance vessel morphology.

Finally, the limited data available suggest that, contrary to the essential form, hypertension secondary to renovascular disease could promote media growth in human small subcutaneous arteries [48–51].

There are at least two caveats regarding the interpretation of these clinical data. First, the extent to which they might be contaminated by the aforementioned sampling problem is impossible to assess. Second, the subcutaneous vasculature is not necessarily representative of other vascular beds. There are a few observations to mitigate the latter concern. We may recall here the evidence of eutrophic remodeling in the intestinal microcirculation of hypertensive patients. In addition, a positive correlation has been found in hypertensive patients between coronary flow reserve and the M/L ratio of subcutaneous arteries, indeed supporting that hypertensive changes of microvascular structure were not limited to the subcutis [52, 53]. Finally, Harazny et al. [50] have very recently been able to evaluate the vascular remodeling of retinal arterioles in patients with treated hypertension and without advanced retinopathy (stage III or IV). To that effect, they used laser Doppler imaging whereby outer and inner diameters were, respectively, determined from reflection and perfusion images. Results indicated a higher ratio when blood pressure control was poorer than when it was satisfactory.

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Clinical Study

Endothelial Activation Microparticles and Inflammation Status Improve with Exercise Training in African Americans

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African Americans have the highest prevalence of hypertension in the world which may emanate from their predisposition to heightened endothelial inflammation. The purpose of this study was to determine the effects of a 6-month aerobic exercise training (AEXT) intervention on the inflammatory biomarkers interleukin-10 (IL-10), interleukin-6 (IL-6), and endothelial microparticle (EMP) CD62E+ and endothelial function assessed by flow-mediated dilation (FMD) in African Americans. A secondary purpose was to evaluate whether changes in IL-10, IL-6, or CD62E+ EMPs predicted the change in FMD following the 6-month AEXT intervention. A pre-post design was employed with baseline evaluation including office blood pressure, FMD, fasting blood sampling, and graded exercise testing. Participants engaged in 6 months of AEXT. Following the AEXT intervention, all baseline tests were repeated. FMD significantly increased, CD62E+ EMPs and IL-6 significantly decreased, and IL-10 increased but not significantly following AEXT. Changes in inflammatory biomarkers did not significantly predict the change in FMD. The change in $VO_{2\,max}$ significantly predicted the change in IL-10. Based on these results, AEXT may be a viable, nonpharmacological method to improve inflammation status and endothelial function and thereby contribute to risk reduction for cardiovascular disease in African Americans.

1. Introduction

The most recent report (May 2012) from the World Health Organization, as well as the preponderance of published articles on hypertension and race, supports the conclusion that African Americans have the highest prevalence of hypertension in the world. Research has demonstrated that African Americans have a greater prevalence of endothelial dysfunction when compared to their Caucasian counterparts, and researchers report that they suspect that this predisposes them to hypertension [1, 2]. Hypertension is a result of independent and interactive effects from multiple genetic and

environmental factors. Inflammation of the endothelium, a pathological mechanism that can cause endothelial dysfunction and a precursor to hypertension, has been identified as one of these factors.

It is thought that the balance between pro- and anti-inflammation plays a crucial role as a determinant of endothelial homeostasis and health [3]. Bautista reviewed multiple studies and reported a positive association between hypertension and some proinflammatory markers including C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) [4]. Experimental evidence has also established that several proinflammatory cytokines, including IL-6, contribute to endothelial dysfunction which may lead to increased peripheral vascular resistance and consequently hypertension [5]. In contrast, interleukin-10 (IL-10) is a multifunctional cytokine that inhibits activation and the effector function of T cells, monocytes, and macrophages and ultimately terminates inflammatory responses [6]. Elevated circulating levels of IL-10 are associated with improved endothelial function in individuals with ongoing systemic inflammation [7] and in coronary artery disease (CAD) patients [3].

Brachial artery flow-mediated dilation (FMD) is the conventional method used to assess endothelial function and health in humans because of its high feasibility as a noninvasive, ultrasound testing modality. Its evaluation is thought to be an important index in subjects at risk for cardiovascular disease (CVD) that may contribute to understanding the extent of the inflammatory status of the endothelium [8]. In recent years, evidence suggests that endothelial activation, characterized by increased inflammation, is an early event in endothelial dysfunction and may be identified with the endothelial microparticle (EMP) inducible marker CD62E+ which is sensitive to endothelial activation [9]. Experimental evidence as reported in a review article by Boulanger et al. suggests that plasma levels of EMPs may be a specific marker of endothelial dysfunction in patients with CVD and may provide further information regarding the status of the endothelium beyond vasodilation [10]. Therefore, the detection and quantification of EMPs may be a valuable tool to assess cardiovascular risk.

Increased blood flow shear stress during aerobic exercise has been associated with favorable endothelial adaptations [11]. Additionally, chronic aerobic exercise has been demonstrated to improve the plasma inflammatory status, including IL-6 and IL-10, in certain populations. Aerobic exercise training (AEXT) may lead to the adaptive response of increasing plasma IL-10 concentrations and decreasing both plasma IL-6 concentrations and CD62E+ EMPs in African Americans, thereby improving endothelial function by reducing inflammation. However, despite the high prevalence of hypertension and CVD in African Americans, few studies have investigated the effects of AEXT on inflammation and endothelial health in an effort to develop preventive measures to reduce the CVD disease burden among this high-risk population. Therefore, the purpose of this study was to determine the effects of a 6-month AEXT intervention on plasma levels of IL-10, IL-6, and CD62E+ EMPs and endothelial function assessed by FMD in a cohort of middle-to-older-aged African Americans. Furthermore, a secondary purpose was to evaluate whether changes in IL-10, IL-6, or CD62E+ EMPs predicted the change in FMD following the 6-month AEXT intervention.

2. Methods

This study employed a pre-post design following the completion of screening and dietary stabilization. Sedentary, putatively healthy, middle-to-older-aged (40–75 y/o) African American men and women were recruited and underwent a series of screening tests to ensure that they were free of disease and conditions that may confound interpretation of results. All qualified participants then completed a dietary stabilization period in order to control for the effects of interindividual variations in dietary intake. Finally, any participants using antihypertensive monotherapy were appropriately tapered from their medication, and suspension of medication was continued for the duration of the study. This was done to avoid an AEXT by medication interactive effect. Following dietary stabilization and a minimum of 2 weeks after medication tapering, baseline testing was conducted. This included office blood pressure measurements, FMD studies, fasting blood sampling, and graded exercise testing. FMD studies and fasting blood sampling were conducted on separate days but under the same conditions. Upon completion of baseline testing, participants engaged in a 6-month AEXT intervention under the direct supervision of laboratory personnel. At the conclusion of the 6-month intervention, participants repeated all baseline tests.

2.1. Participants. Participants were required to be between the ages of 40–75 years inclusively, sedentary (self-reported, regular aerobic exercisers $\leq$ 2 days per week), nondiabetic (fasting blood glucose $\leq$ 126 mg/dL), nonsmoking ($\geq$2 years), have a clinic blood pressure <160/100 mmHg (i.e., not stage II hypertensive), and have no documented history of CVD, hypercholesterolemia (total cholesterol > 240 mg/dL), renal disease, or pulmonary disease. Participants on lipid lowering medications, medications that affect cardiovascular or renal hemodynamics, or who were taking more than one antihypertensive medication were excluded from this study. Both premenopausal and postmenopausal (self-reported absence of menses) women were included in the study. All postmenopausal women were required to continue their hormone replacement therapy, either on or off, for the duration of the study. These inclusion criteria were used to create a more homogeneous group of middle-to-older-aged African Americans who were at low-to-moderate risk for CVD but who were otherwise putatively healthy. Each participant gave written informed consent following a complete explanation of the study during their first laboratory visit. The protocol was approved by the Temple University Institutional Review Board.

2.2. Screening. Eligibility of all qualified participants was ensured via completion of three screening visits prior to

inclusion in the study. Screening visit one followed a 12-hour postabsorptive single blood sampling to assess blood chemistries and a urinalysis to assess renal function. Any individual with a total cholesterol >240 mg/dL or fasting blood glucose >126 mg/dL was excluded from the study. Estimated glomerular filtration rate (eGFR) was calculated using the four-variable modification of diet in renal disease (MDRD) study equation specific to African Americans. Any participant who exhibited evidence of renal disease (eGFR < 60 mL min^{-1} per 1.73 m^2) was excluded from the study.

Screening visits two and three required all qualified participants to undergo a physician-administered physical examination and a cycle ergometer echocardiogram stress test to confirm that participants displayed no evidence of latent cardiovascular, pulmonary, or other chronic diseases.

2.3. Plasma IL-10 and Plasma IL-6 Concentration. Blood samples were collected in the morning following a 12-hour overnight fast. Blood was drawn into EDTA tubes, centrifuged at 2,000 g for 20 minutes at 4°C, and then the plasma was frozen at −80°C until the time of the assay. Concentrations of IL-10 and IL-6 were determined using an enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN, USA). Assays were conducted and analyzed according to manufacturer's protocol. Absorbance was recorded using a Spectra Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The plate was read at 490 nm with correction for optical imperfections at 650 nm for IL-10 and at 450 nm with correction for optical imperfections at 540 nm for IL-6. Intraassay and interassay CVs were 5.5% and 11.9%, respectively, for IL-10 and 7.4% and 4.5%, respectively, for IL-6.

2.4. CD62E+ Endothelial Microparticles Identification and Quantification. Circulating EMPs were quantified using a venous blood sample obtained from the antecubital vein in the morning following a 12-hour overnight fast. Samples were collected into EDTA tubes using a 21-gauge needle and were centrifuged at 2,000 g for 20 minutes at 4°C immediately after collection to separate plasma from whole blood. Plasma samples were then stored at −80°C until measurement. On the day of analysis, two sequential centrifugation steps were used to reduce background signals contributed by plasma proteins and residual contaminating/unwanted cells and to concentrate microparticles in order to improve the signal-to-noise ratio during flow cytometric analysis. First, plasma samples were thawed and centrifuged at 1,500 g for 20 minutes at room temperature to obtain platelet poor plasma (PPP). The top two-thirds volume of PPP were then transferred to a new tube and further centrifuged at 1,500 g for 20 minutes at room temperature to obtain cell-free plasma. The supernatant was used for microparticle analysis. A volume of 100 μL supernatant was incubated with fluorochrome-labeled antibodies for 20 minutes at room temperature in the dark and then was fixed by adding 93 μL of 10% formaldehyde. The mixture was protected from light and incubated while being gently mixed using a shaker for 20 minutes. The antibody CD62E-PE (15 μL per sample) was used to distinguish EMP subpopulations. All antibodies were obtained from BD Biosciences. After antibody incubation, samples were diluted with 500 mL of 0.22 μm double-filtered PBS before flow cytometric analysis. Two additional samples were also prepared to serve as negative controls and as a calibration. For the negative control tube, 733 μL of PBS was added to one tube. The calibrator sample was prepared using two drops of 0.9 μm standard precision NIST traceable polystyrene particle beads (Polysciences Inc, Warrington, PA, USA), and was added to PBS according to the manufacturer's instructions. All samples were immediately analyzed by flow cytometry.

Samples and controls were analyzed using a BDLSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and BD FACSDIVA software (v 1.2.6; BD Biosciences). Forward scatter scale, side scatter scale, and each fluorescent channel were set in logarithmic scale. Events included in the set gate (<1.0 μm) were identified in forward and side scatter intensity dot representation and plotted on 2-color fluorescence histograms. CD62E+ events <1.0 μm were defined as EMPs. Fluorescence minus one control and nonstained samples were used to discriminate true events from noise and to increase the sensitivity for microparticle detection for each sample. The flow rate was set on medium, and all samples were run for 180 seconds. Using beads, medium flow rate was calculated, and a mean sample volume of 101 μL per 180 seconds was processed. EMPs were expressed as events per μL plasma.

2.5. Brachial Artery Flow-Mediated Dilation. FMD was measured as a percent difference between the diameter of the brachial artery during basal conditions and the diameter of the artery following reactive hyperemia. Brachial artery diameter was measured in response to increased flow. All measurements were performed in the morning following a 12-hour overnight fast during which time participants refrained from food, drink (with the exception of water), caffeine, alcohol, antihistamines, and anti-inflammatory medications. A 7.5 MHz linear phased array ultrasound transducer attached to a Sonos 5500 ultrasound machine (Philips Medical Systems, Bothell, WA, USA) was used to image the brachial artery longitudinally. An electrocardiogram (ECG) was continuously monitored. All measurements of brachial artery diameter and blood velocity were measured by a trained cardiologist after the participant rested in a quiet and dim room at a controlled ambient temperature of 20–26°C for a minimum duration of 10 minutes. The participant's right arm was comfortably immobilized in the extended position to allow for ultrasound scanning of the brachial artery 5–10 cm above the antecubital fossa. Simultaneous doppler measurements for blood velocity and 2D ultrasound imaging for right brachial artery diameter were continuously recorded for 2 minutes at baseline. After recording of baseline images, reactive hyperemia was induced by distal occlusion of the vessel using a cuff inflated to a suprasystolic pressure (200 mmHg) for 5 minutes on the right forearm and distal to the antecubital fossa. Brachial artery diameter was then recorded at 1-minute postcuff release at a fixed distance from an anatomic marker at the end of diastole.

2.6. Aerobic Exercise Training Intervention. A submaximal graded exercise test was performed to determine participants' cardiovascular fitness and to develop individualized exercise prescriptions for the AEXT intervention. A modified Bruce protocol submaximal treadmill exercise test was performed with continuous measurement of breath-by-breath gas sampling oxygen consumption (VO_2) using a calibrated metabolic cart (Vmax Encore, SensorMedics, Yorba Linda, CA, USA). ECG was continuously monitored, and the treadmill test was terminated when the participant reached 75–80% of their predicted heart rate reserve. A standard regression formula using data collected by indirect calorimetry (VO_2 averaged over each 60-second period) and ECG (minute heart rates) was used to predict $VO_{2\,max}$, a measure of cardiovascular fitness, as recommended by the American College of Sports Medicine Guidelines for Exercise Testing and Prescription.

Participants engaged in a 24-week AEXT intervention under direct supervision of lab personnel 3x/week, beginning with 20 minutes of exercise/session at 50% of $VO_{2\,max}$. Training duration was then increased by 5 minutes each week until 40 minutes of exercise at 50% of $VO_{2\,max}$ was reached. Training intensity was then increased by 5% each week until 65% of $VO_{2\,max}$ was achieved. At week 8, participants reached the desired exercise duration and intensity of 40 minutes at 65% of $VO_{2\,max}$, which they maintained as their prescription for the remainder of the study. Exercise modes included treadmill walking/jogging, stair stepping, stationary cycling, rowing ergometry, arm ergometry, and elliptical cross-training. To monitor exercise intensity, participants were instructed on how to use heart rate monitors. Study personnel recorded participants' exercise mode, heart rate, and duration in printed logs to ensure adherence to the prescribed exercise training program. Heart rate was recorded every 10 minutes. At week 12, participants completed a second submaximal treadmill exercise test as a basis for adjustment of their exercise prescription to account for changes in cardiovascular fitness. The gradual progression of training duration and intensity was used in order to avoid excessive fatigue and musculoskeletal complaints, thereby maximizing adherence.

2.7. Statistical Analyses. Among the 42 participants who completed the 6-month AEXT intervention, the data used in the statistical analysis for each primary outcome variable were FMD testing ($n = 26$), CD62E+ EMPs ($n = 28$), IL-6 ($n = 32$), and IL-10 ($n = 26$). The differences in each variable sample size are related to issues with participant scheduling, acquiring blood samples, or assay procedure.

Data are expressed as mean ± the standard error of the mean (SEM). The distribution of all variables was examined using the Shapiro-Wilk test of normality. Pre-AEXT and post-AEXT were compared using the paired samples Wilcoxon signed-rank test. Simple linear regression was used to calculate relationships between the variables. Statistical significance was set at a P value of <0.05. All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

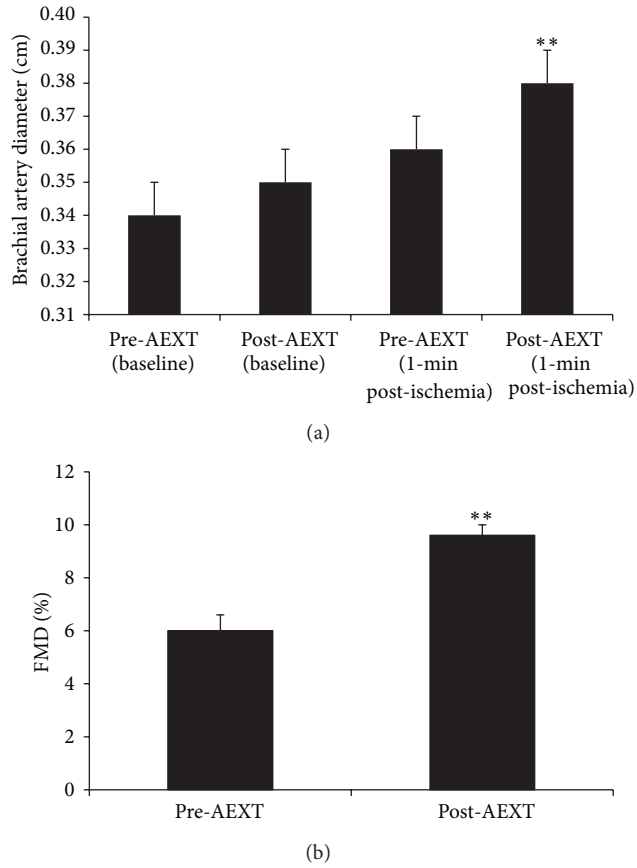

Figure 1: Measures of brachial artery diameter and endothelial function before and after AEXT. The upper panel (a) shows brachial artery diameter at baseline and at 1-minute post-ischemia pre- and post-AEXT. The lower panel (b) shows FMD% pre- and post-AEXT. Bars are expressed as mean ± SEM. ** Denotes significant differences pre- versus post-AEXT; $P < 0.01$.

3. Results

3.1. Laboratory Values of Participants before and after Exercise Training. The study group consisted of 42 African American men ($n = 6$; 14.3%) and women ($n = 36$; 85.7%). The mean age of the group was 52.7 ± 1.0 years. The laboratory values of the participants measured prior and subsequent to the AEXT intervention are presented in Table 1. The 6-month AEXT intervention significantly increased $VO_{2\,max}$ and significantly decreased BMI, plasma triglycerides, and fasting blood glucose. Total cholesterol, LDL cholesterol, HDL cholesterol, and mean systolic and diastolic blood pressure were not significantly changed following the AEXT intervention.

3.2. Endothelial Function before and after Exercise Training. Pre- and post-AEXT values of measures obtained from assessment of endothelial function by FMD testing are presented in Figure 1. There was a 2.9% increase in baseline brachial artery diameter (Figure 1(a)) following AEXT; however, this increase was not statistically significant. Brachial artery diameter 1-minute post-ischemia was significantly

Table 1: Laboratory values of participants before and after AEXT.

Variable	Participant number	Pre-AEXT	Post-AEXT	Percent change
BMI (kg/m^2)	$n = 42$	31.4 ± 0.9	30.6 ± 0.9*	−2.5%
$VO_{2\,max}$ (mL/kg/min)	$n = 41$	25.9 ± 0.9	28.2 ± 1.1**	8.9%
SBP (mm Hg)	$n = 41$	124.2 ± 1.9	123.6 ± 2.2	−0.5%
DBP (mm Hg)	$n = 41$	78.7 ± 1.1	78.9 ± 1.2	0.3%
Total cholesterol (mg/dL)	$n = 35$	190.9 ± 4.2	190.4 ± 5.2	−0.3%
LDL cholesterol (mg/dL)	$n = 36$	108.7 ± 3.6	111.9 ± 4.3	2.9%
HDL cholesterol (mg/dL)	$n = 36$	66.8 ± 3.3	65.6 ± 3.4	−1.8%
Triglycerides (mg/dL)	$n = 36$	83.0 ± 5.7	70.1 ± 3.3**	−15.5%
Fasting glucose (mg/dL)	$n = 34$	95.1 ± 1.7	88.5 ± 1.8**	−6.9%

Participant number represents usable sample for variables.
Values are expressed as mean ± SEM. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein.
*Denotes significant differences pre- versus post-AEXT; $P < 0.05$.
**Denotes significant differences pre- versus post-AEXT; $P < 0.01$.

increased by 5.6% (Figure 1(a)) following AEXT. The relative increase in brachial artery diameter from baseline to post-ischemia (FMD%) was significantly increased by 60% (Figure 1(b)) following AEXT.

3.3. Inflammatory Biomarkers before and after Exercise Training. Pre- and post-AEXT values for the inflammatory biomarkers are presented in Figure 2. The 6-month AEXT intervention elicited statistically significant changes in CD62E+ EMPs and IL-6. There was a significant 47.3% decrease in CD62E+ EMPs (Figure 2(a)) and a significant 12% decrease in IL-6 (Figure 2(b)) following AEXT. IL-10 was increased by 4.9% (Figure 2(c)) following AEXT; however, it was not statistically significant.

3.4. Regression Analyses. Simple linear regression using change values for each biomarker revealed that changes in CD62E+ EMPs, IL-10, or IL-6 did not significantly predict the change in FMD%. Based on the combined r^2 values, CD62E+ EMPs, IL-10, and IL-6 accounted for 10.3% of the change in FMD%. Simple linear regression demonstrated that the change in $VO_{2\,max}$ significantly predicted the change in IL-10 ($n = 25$; $P = 0.02$).

4. Discussion

The primary findings of the present study demonstrated that 6 months of AEXT elicited significant positive improvements in the inflammatory biomarkers IL-6 and CD62E+ EMPs, as well as the endothelial function marker FMD in a cohort of middle-to-older-aged African Americans. Other studies that measured inflammatory biomarkers and endothelial function prior to and subsequent to AEXT have demonstrated similar results, but to our knowledge this is the first study that measured all of these complementary biomarkers prior to and subsequent to AEXT in an African American population.

Improvements in FMD following AEXT have been well documented in previous research. Cornelissen et al. demonstrated a significant increase in FMD% following 12 weeks of aerobic exercise in stable CAD patients [12]. Similarly, Luk et al. conducted research to determine the effect of 8 weeks of AEXT on FMD in patients with stable CAD and demonstrated significant improvements in FMD [13]. Furthermore, Nualnim et al. reported that 12 weeks of regular swimming exercise in a group of putatively healthy adults with prehypertension or stage 1 hypertension significantly improved FMD [14]. However, none of these published studies included sufficient numbers of African Americans to draw any conclusions about the effect of AEXT on FMD in this population. The present study provides some evidence that AEXT may also be beneficial for improving FMD in African Americans.

The results of the present study demonstrated that the change in inflammatory biomarkers CD62E+ EMPs, IL-6, and IL-10 together accounts for 10.3% of the change in FMD% following AEXT. These findings suggest that the three inflammatory biomarkers measured may be contributory to the health of the endothelium; however, there are other factors that may also impact overall endothelial health. It is possible that other biomarkers that were not the focus of this study such as C-reactive protein, oxidized LDL, vascular adhesion molecule, or von Willebrand factor may be better predictors of the change in FMD% with AEXT in this population.

To our knowledge, the effect of AEXT on CD62E+ EMPs has not been previously investigated in any population. CD62E+ EMPs have been identified as markers of inflammatory endothelial cell activation [9, 10, 15, 16]. Therefore, the detection and quantification of EMPs may be a valuable marker in the early detection of cardiovascular risk. Lee et al. demonstrated that a high level of CD62E+ EMPs is associated with cardiovascular events in patients with a history of stroke, suggesting that systemic endothelial activation is associated with the risk for cardiovascular morbidities [17]. The present study provides some of the first evidence that AEXT may

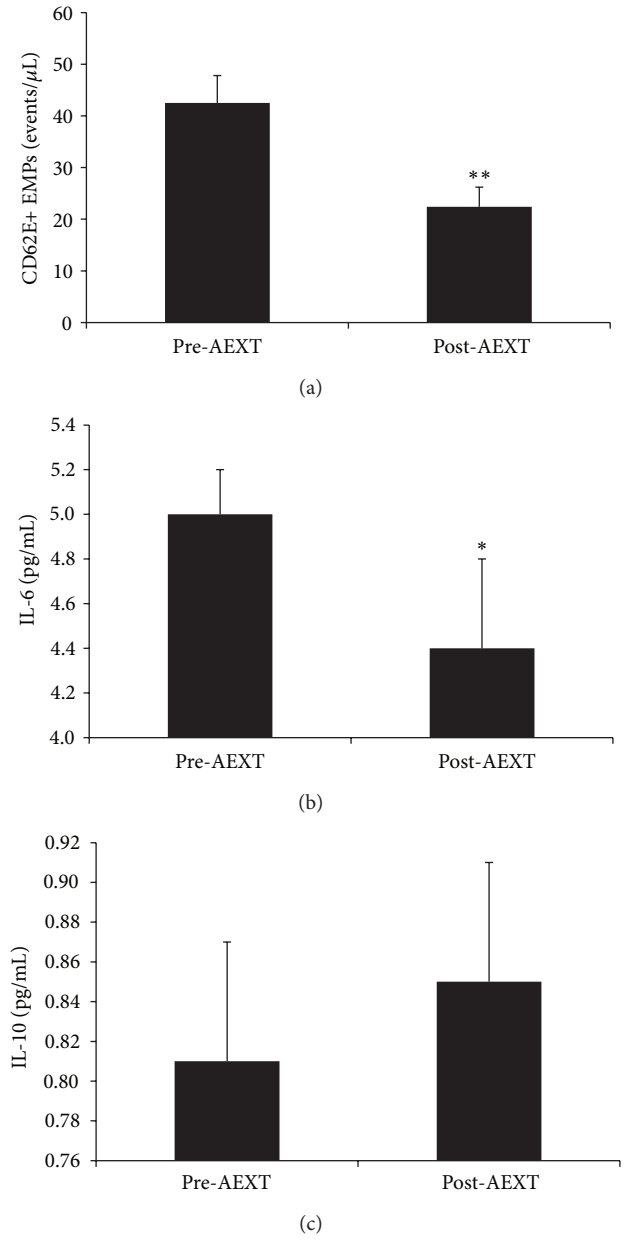

Figure 2: Inflammatory biomarkers before and after AEXT. The upper panel (a) shows CD62E+ EMPs pre- and post-AEXT. The middle panel (b) shows IL-6 pre- and post-AEXT. The lower panel (c) shows IL-10 pre- and post-AEXT. Bars are expressed as mean ± SEM. *Denotes significant differences pre- versus post-AEXT; $P < 0.05$. **Denotes significant differences pre- versus post-AEXT; $P < 0.01$.

attenuate endothelial activation in African Americans which may have clinical importance given the recent findings from Lee et al.

IL-6 is a pleiotropic cytokine whose primary biological functions include mediation of proinflammatory responses and cytoprotection [18]. It is released by endothelial cells in response to inflammatory stress and is essential in the pathogenesis of vascular inflammatory diseases [19, 20]. A review of multiple studies by Boos and Lip concluded that IL-6 contributes to endothelial dysfunction which may lead to increased peripheral vascular resistance and consequently hypertension [5]. The effect of AEXT on circulating levels of IL-6 following AEXT has been previously investigated. Beckie et al. demonstrated that there were significant reductions in IL-6 concentrations in women with CAD following a cardiac rehabilitation exercise program [21]. Additionally, a meta-analysis by Swardfager evaluated changes in inflammatory biomarkers subsequent to exercise interventions in patients with CAD and demonstrated significant decreases in plasma IL-6 concentration [22]. The present study provides further evidence that AEXT may attenuate plasma IL-6 concentrations, and to our knowledge, demonstrating such for the first time in an African American population.

IL-10 is an anti-inflammatory cytokine produced by immune and nonimmune cells [23]. Its primary biological function is to attenuate inflammatory responses, and it has an anti-inflammatory effect on monocyte/endothelium interactions [6, 24]. IL-10 has been demonstrated to participate in preserving endothelial function during acute inflammation [25]. Although a clear mechanism has not yet been elucidated, emerging evidence suggests that IL-10 has a role in vascular protection [23]. Most studies that have measured serum or plasma IL-10 concentrations have found detectable levels of IL-10 in a diseased population versus healthy subjects. Blay et al. measured IL-10 in 153 subjects with non-Hodgkin's lymphoma and compared them to a control group of 60 healthy subjects. The researchers found that IL-10 was not detectable in any of the healthy subjects, but it was detectable in about half of the diseased subjects [26]. In the present study, the fact that we were able to detect IL-10 even at relatively low levels in our putatively healthy African American population may be interpreted as a result of the increased predisposition in this population to chronic low-grade inflammation.

Several studies have previously examined the effect of AEXT on circulating levels of IL-10. Ribeiro et al. examined the effect of AEXT on the plasma inflammatory status of post-myocardial infarction patients and concluded that AEXT increased IL-10, suggesting enhancement of anti-inflammation [27]. Furthermore, Goldhammer et al. demonstrated that AEXT in CAD patients was effective in increasing IL-10, leading to improvements in coronary risk status [28]. In a review article by Batista et al. on multiple studies of exercise and IL-10, the authors concluded that the anti-inflammatory effect induced by AEXT seems primarily to be mediated by IL-10 [29]. In the present study, the effect of AEXT on circulating levels of IL-10 was investigated, to our knowledge, for the first time in an African American population, and the results demonstrated that there was a trend for increased IL-10 subsequent to AEXT, although statistical significance was not achieved. More notably, the results of the present study indicated that in our African American population sample, the significant improvement in cardiovascular fitness, as measured by $VO_{2\,max}$, was related to the improvement in plasma levels of IL-10. Future research is warranted in order to assess whether further increases in $VO_{2\,max}$, subsequent to AEXT, elicit a significant improvement in IL-10.

We previously reported that African American endothelial cells had significantly greater levels of IL-6 protein expression and produced greater amounts of IL-6 in response to TNF-α, an inflammatory cytokine [30]. Oxidative stress and inflammation often occur simultaneously [31]. Kalinowski demonstrated that African Americans have increased levels of oxidative stress resulting in endothelial dysfunction when compared to Caucasians [32], and we also previously demonstrated that compared to Caucasian endothelial cells, African American endothelial cells had significantly greater protein expression levels of NADPH oxidase, the principal source of reactive oxygen species in endothelial cells [33]. Together, the work of others and our group suggests a heightened inflammatory and oxidative stress status in African American endothelial cells. Therefore, an intervention that can dampen this condition before endothelial dysfunction develops to the point where it is manifested clinically may be very important. The results from our present study extend upon our previous work that AEXT may be a nonpharmacological treatment modality which may improve endothelial health in middle-to-older-aged African American adults free of overt CVD.

The positive changes in endothelial and inflammatory biomarkers after AEXT demonstrated in this study may indicate considerable improvement in CVD risk for the African American population. A substantial portion of the CVD risk reduction associated with exercise training cannot be entirely explained by changes in conventional CVD risk factors [34]. It has been suggested that direct effects of exercise on the vessel wall may account for some of the remaining risk factor reduction gap [35].

The participants in the present study had no significant changes in mean blood pressure following AEXT. These findings are in agreement with most studies that measured blood pressure subsequent to AEXT in individuals with relatively normal resting blood pressure levels. In studies on normotensive and/or prehypertensive populations, blood pressure did not significantly change following AEXT in most cases [36, 37]. Conversely, a review by Hagberg et al. reported that blood pressure significantly decreased in 75% of the hypertensive subjects following AEXT [38]. Despite the fact that mean blood pressure did not change in the present study, the endothelial and inflammatory biomarkers measured in this study related to endothelial health and CVD improved considerably. Therefore, the pronounced benefits on CVD risk reduction resulting from AEXT may go beyond simple blood pressure reduction in an African American population as elucidated by the results of the present study.

Several limitations must be noted when interpreting our study findings. First, our sample size is small, but this was due to the exclusion of diabetics, smokers, participants with CVD, or other chronic diseases and those on medications that affect cardiovascular or renal hemodynamics, on lipid lowering medications, or on more than one antihypertensive medication. This was done to create a more homogenous group and to ensure lack of confounding variables that may influence endothelial or inflammatory marker levels. It should be noted that even with a relatively small sample size, we observed significant changes in three of the four primary outcome measures subsequent to AEXT. Second, because of the observational nature of the study design, we cannot infer mechanisms underlying exercise training induced changes in inflammatory status or endothelial function. Third, there are presently no standardized methods for the measurement of microparticles. Processing and analyzing techniques differ from investigator to investigator, and thus comparisons across studies for EMPs should be done cautiously. Fourth, no control group was included in the study design, and thus it is difficult to ascertain whether the observed changes were exclusively due to AEXT and not to the result of an unidentified confounding factor. Finally, the sample population was predominately female, and thus our findings may have limited generalizability to African American males.

In conclusion, the results of the present study are novel because to our knowledge, for the first time, FMD%, CD62E+ EMPs, IL-6, and IL-10 have been measured together prior to and subsequent to AEXT in a population of African Americans. The primary findings of the study revealed favorable alterations in the endothelial and inflammatory biomarkers measured subsequent to AEXT. Therefore, aerobic exercise training may be a viable, nonpharmacological method to improve inflammation status and endothelial function and thereby contribute to risk reduction for CVD in African Americans.

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Research Article

Strategies Aimed at Nox4 Oxidase Inhibition Employing Peptides from Nox4 B-Loop and C-Terminus and p22^{phox} N-Terminus: An Elusive Target

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Although NADPH oxidase 4 (Nox4) is the most abundant Nox isoform in systemic vascular endothelial and smooth muscle cells, its function in the vascular tissue is not entirely known. The literature describes a pathophysiological role for Nox4 in cardiovascular disease; however, some studies have reported that it has a protective role. To date, specific Nox4 inhibitors are not available; hence, the development of a pharmacologic tool to assess Nox4's pathophysiological role garners intense interest. In this study, we selected peptides corresponding to regions in the Nox4 oxidase complex critical to holoenzyme activity and postulated their utility as specific competitive inhibitors. Previous studies in our laboratory yielded selective inhibition of Nox2 using this strategy. We postulated that peptides mimicking the Nox4 B-loop and C-terminus and regions on p22^{phox} inhibit Nox4 activity. To test our hypothesis, the inhibitory activity of Nox4 B-loop and C-terminal peptides as well as N-terminal p22^{phox} peptides was assessed in a reconstituted Nox4 system. Our findings demonstrate that Nox4 inhibition is not achieved by preincubation with this comprehensive array of peptides derived from previously identified active regions. These findings suggest that Nox4 exists in a tightly assembled and active conformation which, unlike other Noxes, cannot be disrupted by conventional means.

1. Introduction

NADPH-oxidase- (Nox-) derived reactive oxygen species (ROS) play a central role in the destruction of pathogenic organisms by phagocytes. The phagocyte Nox complex is composed of flavocytochrome b558, an integral membrane heterodimer composed of gp91^{phox} (a.k.a. Nox2) and p22^{phox}, four cytoplasmic protein subunits, p47^{phox}, p67^{phox}, p40^{phox}, and the regulatory low molecular weight GTPase Rac. In resting phagocytes, the Nox enzyme is in a dormant state. Upon activation, the cytosolic Nox subunits translocate to Nox2 and p22^{phox} followed by the transfer of one electron from NADPH to molecular oxygen, resulting in the formation of superoxide anion ($O_2^{\bullet -}$) and microbicidal activity [1].

Nox2 is also expressed in cells other than phagocytes, [2, 3] and excessive ROS generation by nonphagocytic Nox2 contributes to a wide variety of disorders [3–5]. Over the past decade since the discovery of Nox2 homologs Nox1, Nox3, Nox4, Nox5, DUOX1 and DUOX2 [4], interest has greatly increased in Nox enzymes and the development of isoform-specific Nox inhibitors. Although numerous chemical compounds have been shown to inhibit Nox enzymes, none of these to our knowledge is specific for one isoform [6, 7]. Importantly, rationally designed, sequence-specific peptide-based inhibitors have the potential to be among the most selective and effective inhibitors of Nox because of their potential to selectively target unique protein interactions within the enzyme. A previous study by our group demonstrated that a peptide sequence mimicking amino acids 86–94 in the first intracellular loop of Nox2 (B-loop) specifically inhibits Nox2 activation *in vitro* [8, 9]. The effectiveness of this peptide to inhibit ROS production *in vivo* has been widely

shown, and has led to its wide use in numerous studies [10–15]. To date, peptidic inhibitors have been reserved for Nox2; that is, no prior studies tested whether inhibition of other homologs can be achieved by this strategy.

Nox4 is the most abundant Nox isoform in endothelial cells, vascular smooth muscle cells, and the kidney [16, 17], but it is also expressed in the heart, central nervous system, airways, and skeletal muscle [4]. Animal and human studies have shown that Nox4 plays an important role in the pathophysiology of a wide variety of disorders, including systemic hypertension [18], diabetes mellitus [19], vascular injury [20], atherosclerosis [21], ischemic stroke [22], pulmonary fibrosis [23], and diabetic nephropathy [24]. Collectively, these data suggest that Nox4 oxidase is a major contributor to oxidative stress in these pathologic conditions, and blocking the undesirable actions of Nox4 could become a therapeutic strategy to attenuate oxidative stress in patients with these disorders. Unlike other Nox isoforms, numerous studies have also described that Nox4 is involved in a variety of physiological processes, including cell differentiation, survival, and migration [16, 25–27]. Moreover, a few studies have reported that Nox4 has a protective role in cardiovascular tissue, although this is still somewhat controversial [19, 28, 29]. Presently, no specific inhibitors of Nox4 (small molecule or peptidic) are available to the scientific community [30] to elucidate the pathophysiological and/or physiological roles of Nox4.

Nox4 oxidase is a unique Nox isozyme as it differs from the usual model of multimeric Nox assembly found in Nox1, Nox2, and Nox3. Indeed, Nox4 does not require interaction with any of the conventional cytosolic Nox subunits for ROS generation and the membrane-bound subunit $p22^{phox}$ is, to date, the only known classical subunit associated with Nox4. Recently, Poldip2 has been described as a modulator of Nox4 [31]; however, in this study we aimed to target the core of the enzyme.

A previous study reported that mutagenesis of arginine residues in the Nox4 B-loop impedes activity of Nox4 [32]. Moreover, it was suggested that the B-loop of Nox4 serves as a binding sequence facilitating interaction of C-terminal NADPH-binding domain of Nox4 with its membrane-spanning region [32]. In addition, deletion of the first 11 amino acids at the $p22^{phox}$ N-terminus completely abrogated Nox4 activity [33]. These data suggest that interaction of Nox4 B-loop with the C-terminal domain as well as association of Nox4 with the N-terminus of $p22^{phox}$ is important for Nox4 activity. We postulated that recombinant excess of Nox4 B-loop and key $p22^{phox}$ N-terminal peptide mimics would disrupt intramolecular B-loop-C-terminal and Nox4-$p22^{phox}$ interactions, respectively, leading to inhibition of Nox4-derived ROS production. Accordingly, the aim of the present study was to investigate whether targeting the Nox4 B-loop and C-terminal domain with sequential 15-mer and nonamer peptide sequences disrupts their interaction and inhibits Nox4 activity. In the present study, $p22^{phox}$ N-terminal tail peptides were also tested for their ability to inhibit Nox4 activity.

2. Materials and Methods

Catalase, diphenyleneiodonium chloride (DPI), flavin adenine dinucleotide (FAD), horseradish peroxidase (HRP), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amplex Red was purchased from Invitrogen (Eugene, OR, USA). Protease inhibitor cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All peptides were synthesized by the Tufts University Core Facility (Boston, MA, USA). The purity of peptides was over 97%. Since the current studies were carried out using COS-Nox4 cell lysates, it is important to note that the peptides used in this study did not require chimeric design containing *tat* peptide for cell permeation.

2.1. Cell Lines and Cell Culture. COS-22 cells (COS-7 cells stably expressing human $p22^{phox}$) were kindly provided by Dr. Mary C. Dinauer (Indiana University, School of Medicine) [34]. COS-22 cells were maintained in Dulbecco's Modified Eagle Medium (Mediatech, Inc., Manassas, VA, USA) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.

2.2. Plasmid Preparation, Amplification, and Purification. Plasmid encoding full-length human cDNAs for Nox4 (pcDNA3-hNox4) was kindly provided by Dr. David Lambeth (Emory University, GA) [35]. For human Nox4 expression, the BglII/NotI restriction fragment from the pcDNA3-hNox4 was subcloned into the plasmid pcDNA3.1/Hygro(−) (Invitrogen, Carlsbad, CA) to generate pcDNA3.1/Hygro-hNox4. The fragment sequence, in-frame insertion, and orientation were validated by DNA sequencing after PCR amplification. pcDNA3.1/Hygro-hNox4 was amplified into *Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA) and purified with a QIA filter plasmid purification kit (QIAGEN Inc., Valencia, CA).

2.3. Transfection. Cell transfection was carried out using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. COS-22 cells were transiently transfected with pcDNA3.1/Hygro-hNox4 (COS-Nox4 cells). Western blot experiments were performed to validate the expression of Nox4 as we reported previously [8]. Twenty-four hours after transfection, cells were harvested by incubating with 0.05% trypsin/EDTA for 5 min at 37°C. Following addition of DMEM/10% FBS to neutralize the trypsin, the cells were pelleted by centrifugation at 1000 ×g for 5 min at 4°C and used for the experiments.

2.4. Hydrogen Peroxide- (H_2O_2-) Generating Activity. H_2O_2 production was quantified in lysed COS-Nox4 cells as described previously [36]. It is important to note that COS-Nox4 cells do not produce $O_2^{\bullet-}$ [8]. COS-Nox4 and COS-22 cells were suspended to a concentration of 5×10^7 cells/mL in ice-cold disruption buffer (PBS containing 0.1 mM EDTA,

10% glycerol, protease inhibitor cocktail, and 0.1 mM PMSF). The cells were lysed by five freeze/thaw cycles and passed through a 30-gauge needle five times to further lyse the cells. Throughout all these procedures, extreme care was taken to maintain the lysate at a temperature close to 0°C. Incubation of COS-Nox4 cell lysate (10 μg/100 μL) with peptides was performed in assay buffer (25 mM Hepes, pH 7.4, containing 120 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 25 μM FAD, 0.1 mM Amplex Red, and 0.32 U/mL of HRP) for 15 or 60 min at room temperature on an orbital shaker (120 movements/min), before the addition of 36 μM NADPH, to initiate H_2O_2 production. This relatively low concentration of NADPH was used because it was found that higher concentrations interfered with Amplex Red fluorescence. Fluorescence measurements were made using a Biotek Synergy 4 Hybrid Multi-Mode Microplate Reader (excitation wavelength: 560 nm; emission wavelength: 590 nm). A standard curve of known H_2O_2 concentrations was developed using the Amplex Red assay (as per the manufacturer's instructions) and was used to quantify H_2O_2 production in lysed COS-Nox4 cells. The reaction was monitored at room temperature for 40 min. The emission increase was linear during this interval. The rate of H_2O_2 production was interpolated from the standard curve and is 609 ± 0.01 nmol H_2O_2/min/mg protein. The effect of a peptide on Nox4-derived H_2O_2 production was expressed as percent inhibition of Nox4, which was calculated by considering H_2O_2 production by control mixtures in the absence of peptide as 100%.

2.5. Statistical Analysis. All results are expressed as means ± SEM. Significance of the differences was assessed by two-way ANOVA followed by Bonferroni post hoc test. $P < 0.05$ was considered to be statistically significant.

3. Results

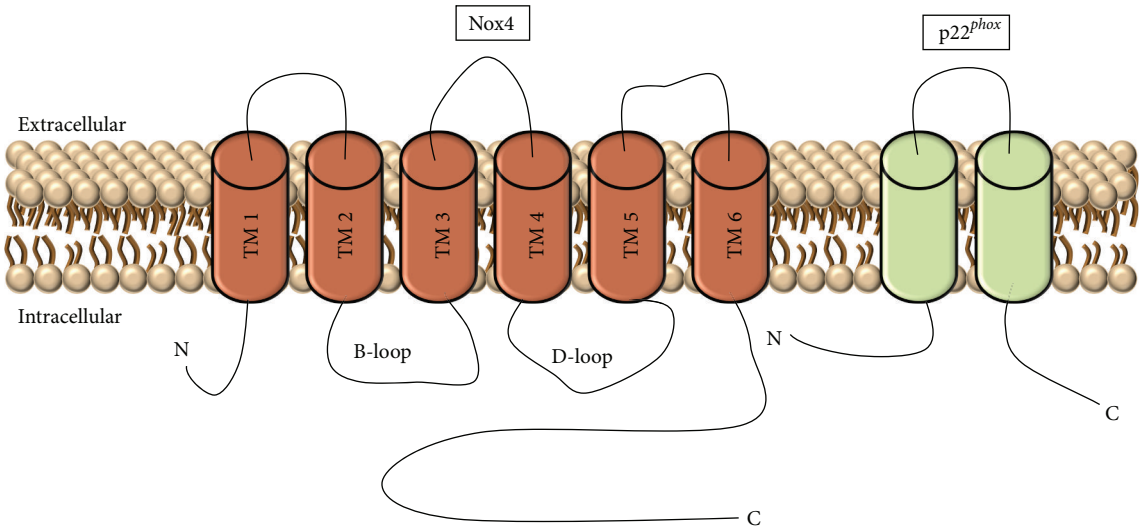
3.1. Nox4 Catalytic Activity Is Not Inhibited by Preincubation with Nox4 B-Loop Peptides. The first intracellular loop of Nox4 (B-loop) is essential for catalytic activity, that is, hydrogen peroxide (H_2O_2) generation, as previously demonstrated by point mutations targeting this region (Figure 1(a)) [32]. Amino acid sequence alignment revealed three subregions of Nox B-loops where two conserved regions flank a highly variable central region (Figure 1(b)). The two flanking conserved regions are shared by all Nox isoforms, whereas the central region is variable across Nox isoforms [32]. Previous data indicated that B-loop peptides bind to the C-terminal of the enzyme and that this interaction is important for activity [32]. We postulated then that this interaction would be competitively blocked by peptides derived from the B-loop, as we showed for Nox2 [8]. In the current study, peptide sequences derived from the variable and conserved regions of Nox4 B-loop were tested for their ability to inhibit Nox4 activity. As shown in Figure 1(c), the rate of H_2O_2 generation was significantly higher in COS-Nox4 cell lysates as compared with nontransfected controls, and ~90% of the Nox4-dependent activity was inhibited by the flavin-containing enzyme inhibitor diphenyleneiodonium (DPI; 50 μM). Detection of H_2O_2 was confirmed by inhibition of fluorescence with catalase (3000 U/mL). As demonstrated in Figure 1(d), peptide B90–98 (0.1–100 μM) did not inhibit Nox4-derived H_2O_2 production. As substitution of Arg-96 to glutamic acid previously resulted in almost complete loss of Nox4 activity [32], we tested whether the R96E mutant version of B90–98 (PSRRTR**E**LL) is capable of inhibiting Nox4 activity. Similar to the wild-type peptide, the mutant peptide did not inhibit Nox4 activity (Figure 1(e)).

Mutation of Arg-84 to alanine on the N-terminal conserved Nox4 B-loop region [32] and replacement of Ser-101 with acidic residues in the C-terminal Nox4 B-loop conserved region were previously shown to inhibit Nox4 [37]. Thus, we tested whether inhibition of Nox4 activity can be achieved by proximal and distal Nox4 B-loop peptides. Our data demonstrated that preincubation of COS-Nox4 cell lysates with neither peptides B77–91, B82–96, nor B92–106 inhibited H_2O_2 production (Figure 1(f)).

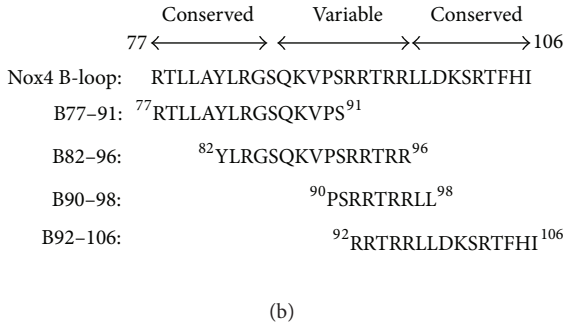
3.2. Nox4 C-Terminal Tail Peptides Do Not Inhibit Nox4 NADPH Oxidase. A previous study demonstrated that the last 22 amino acids of the whole Nox4 protein are critical for catalytic activity (Figure 2(a)) [38]. The presence of charged residues in this flexible region of Nox4 may suggest that electrostatic effects could promote interaction between the C-terminus and B-loop and/or $p22^{phox}$. Thus, three overlapping octameric peptides (C555–562, C560–567, and C565–572) and a nonameric peptide (C570–578) were synthesized to cover the last 22 amino acids of Nox4, and these were tested for their ability to inhibit Nox4 activity. Application of these C-terminal tail peptides did not affect Nox4 activity (Figure 2(b)).

3.3. $p22^{phox}$ N-Terminal Tail Peptides Do Not Inhibit Nox4 NADPH Oxidase. Expression of $p22^{phox}$ is required for Nox4 activity, and, to date, $p22^{phox}$ is the only known classical core protein associated with Nox4 [39]. Mutagenesis studies provided evidence that deletion of a large span of the $p22^{phox}$ C-terminus (terminal 130 amino acids) did not affect Nox4 activity [33]. In contrast, deletion of the first 11 amino acids at the $p22^{phox}$ N-terminus attenuated Nox4 activity. Deletion of the first 5 amino acids did not affect Nox4 activity, suggesting that the N-terminal region of $p22^{phox}$ between amino acids 6 and 11 is sensitive to modification. A peptide sequence between amino acids 6 and 11 (p22 WT 6–11 (WAMWAN)) was thus synthesized and tested for its ability to inhibit Nox4 (Figure 3(a)). As demonstrated in Figure 3(b), peptide p22 WT 6–11 did not inhibit Nox4 activity. Mutation of tryptophans within this sequence to arginine (p22 W6/9R (RAMRAN)) also abolished Nox4 activity [33]. Similar to the native peptide, the p22 W6/9R mutant did not inhibit Nox4-derived H_2O_2 production.

3.4. Increased Incubation Time and Temperature Do Not Facilitate Peptide-Induced Inhibition. Our data demonstrate that preincubation of COS-Nox4 cell lysates with Nox4 B-loop, Nox4 C-terminal tail, and N-terminal $p22^{phox}$ peptides



(a)



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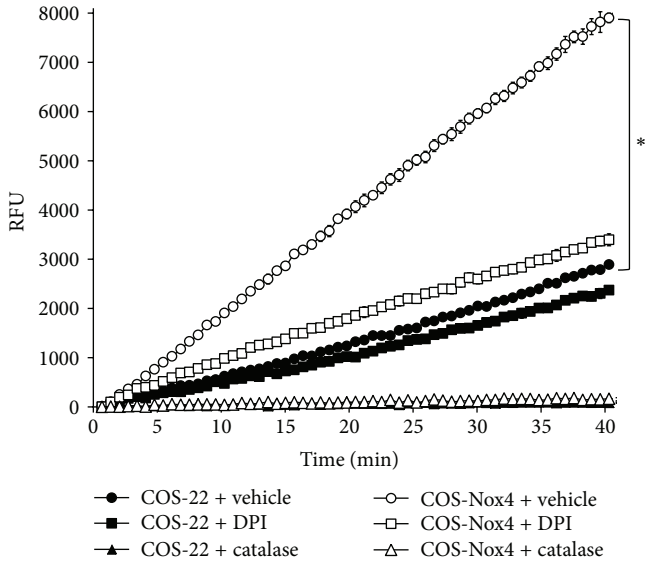

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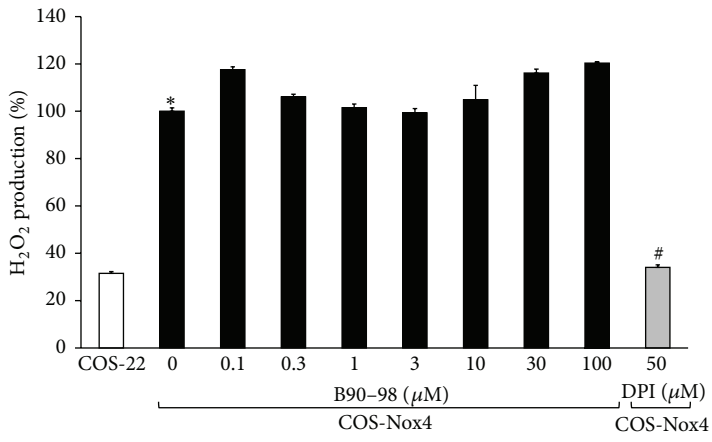
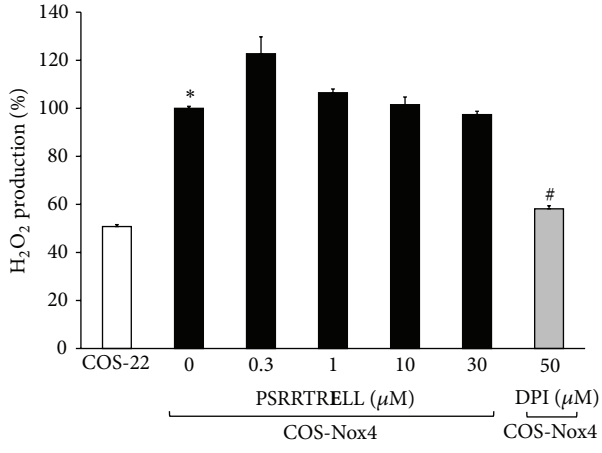
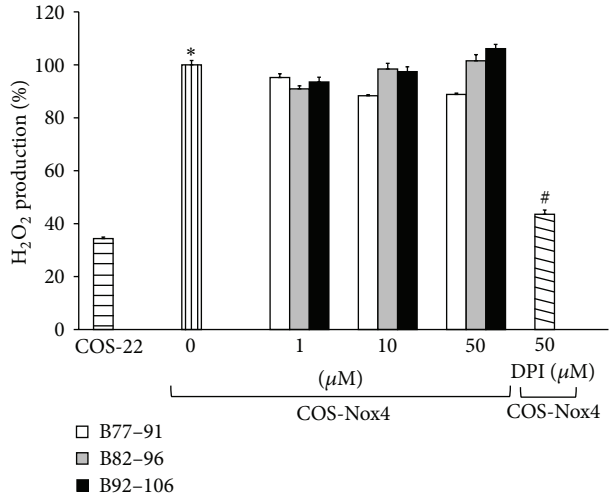


(d)



(e)

Figure 1: Continued.



(f)

Figure 1: Nox4 B-loop peptides and Nox4 activity. (a) A simplified model of Nox4 oxidase with B- and D-loops located intracellularly. $p22^{phox}$, the only known protein required for Nox4 activity, is also shown. (b) Amino acid sequence of Nox4 B-loop and list of overlapping Nox4 B-loop region synthetic 15-mer and nonamer peptides used to target Nox4 activity. The numbers at the N- and C-terminus of each peptide indicate the location of the corresponding residues in the amino acid sequence of Nox4. (c) H_2O_2 generated by Nox4 was measured using Amplex Red fluorescence. H_2O_2 production was initiated by the addition of 36 μM NADPH. The reaction was monitored at 24°C for 40 min. The flavin-containing enzyme inhibitor diphenyleneiodonium (DPI; 50 μM) was used as a positive control. Identification of H_2O_2 was confirmed by inhibition of fluorescence with catalase (3000 U/mL). For comparison, H_2O_2 production in nontransfected COS-22 cell lysate is shown. Data represent the mean ± SEM of 3 experiments. $^*P < 0.05$ indicates significant difference between COS-22 and COS-Nox4 cell lysates. (d) COS-Nox4 cell lysates were preincubated with various concentrations of B90–98 (from 0.1 to 100 μM) for 15 min at 24°C, and H_2O_2 was measured using Amplex Red fluorescence. Data represent the mean ± SEM of 3 experiments. (e) COS-Nox4 cell lysates were preincubated with the R96E mutant version of B90–98 (PSRRTR**E**LL; 0.3 to 30 μM) for 15 min at 24°C, and H_2O_2 was measured. Data represent the mean ± SEM of 3 experiments. (f) COS-Nox4 cell lysates were preincubated with peptides from N- and C-terminal ends of the Nox4 B-loop (B77–91, B82–96, and B92–106; 1 to 50 μM) for 15 min at 24°C, and H_2O_2 was measured. Data represent the means ± SEM of 3 experiments. $^*P < 0.05$ indicates a significant difference between COS-22 and COS-Nox4 cell lysates. $^\#P < 0.05$ indicates a significant difference between COS-Nox4 + 0 μM peptide and COS-Nox4 + DPI.

for 15 min at 24°C did not inhibit Nox4 activity. To allow more time for the peptides to disrupt the targeted domain interactions, we increased incubation time up to 60 min. Our results demonstrated that none of the Nox4 B-loop, Nox4 C-terminal tail, and N-terminal $p22^{phox}$ peptides inhibit Nox4 activity after 60 min of incubation (data not shown). Moving to a new approach, we tested whether providing more kinetic energy, which we proposed to be more favorable for peptide interference with the tightly assembled conformation of Nox4, would facilitate peptide-induced inhibition. Thus, in an attempt to induce temporary perturbations in the structure of Nox4, these experiments were also performed at 37°C. Again, no inhibition of Nox4 was achieved (data not shown).

4. Discussion

This is the first study to our knowledge that seeks to inhibit Nox4 using a peptidic strategy. Indeed, synthetic peptides mimicking key residues in Nox2, $p22^{phox}$, $p47^{phox}$, and Rac1 have been shown to interfere with the activation process of Nox2 and inhibit Nox2-derived $O_2^{\bullet-}$ production [8, 9, 40–43]. Those studies identified domains of functional importance in the assembly of Nox2 oxidase and provided key information about the transformation of the enzyme complex from the dormant, inactive conformation to its active state of the enzyme. Nox1, Nox3, and Duox require cytosolic subunits for activation, while Nox5 does not. Nox4, with $p22^{phox}$, appears to constitutively generate H_2O_2 without the requirement of activating cytosolic subunits, with the exception of Poldip2 [31]. With a desire to target the core of the enzyme, the present study was designed to (a) target multiple residues in the Nox4 sequence with the purpose of determining whether prior information gleaned from mutational studies [32, 33] translates to the potential for peptide mimics disrupting key intramolecular interactions for Nox4 activity and (b) develop isoform-specific peptidic Nox4 inhibitors using such findings.

Previous studies showed that multiple residues in the B-loop and the C-terminal end are critical for the catalytic activity of Nox4 [32, 37]. A recent study using fluorescence polarization demonstrated binding between the Nox4 B-loop and Nox4 dehydrogenase domain and showed that this interaction is weakened by mutation of arginine residues in the B-loop variable region [32]. With this in mind, in the current study, we tested whether application of a peptide mimic (B90–98) from the variable region of Nox4 B-loop interferes with the activity of Nox4. An important premise

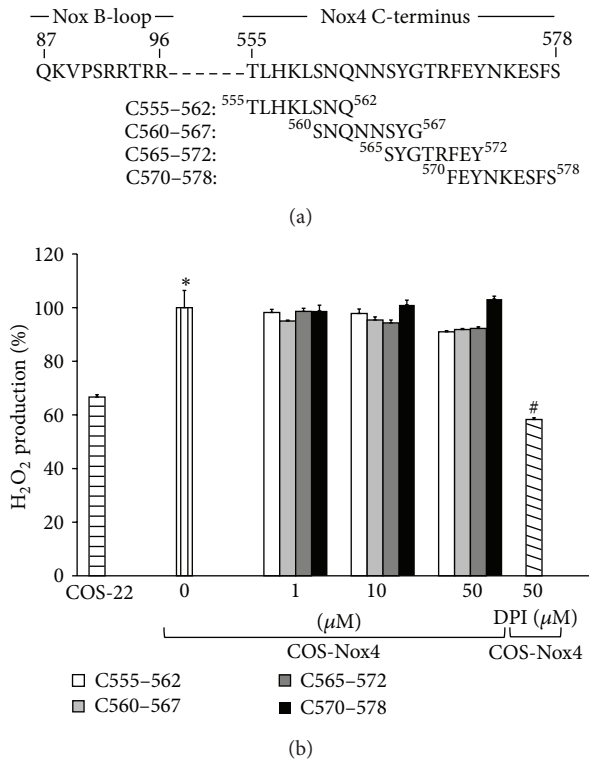


Figure 2: Examination of Nox4 C-terminal tail peptides as Nox4 inhibitors. (a) Map of overlapping Nox4 C-terminal region synthetic octa- and nonapeptides used to inhibit Nox4 activity. Numbers correspond to location and span of the N- and C-terminus for each peptide. (b) COS-Nox4 cell lysates were preincubated with various concentrations of C555–562, C560–567, C565–572, and C570–578 for 15 min at 24°C, and H_2O_2 was measured using Amplex Red. Data represent the mean ± SEM of 3 experiments. $^*P < 0.05$ indicates a significant difference between COS-22 and COS-Nox4 cell lysates. $^\#P < 0.05$ indicates a significant difference between COS-Nox4 + 0 μM peptide and COS-Nox4 + DPI.

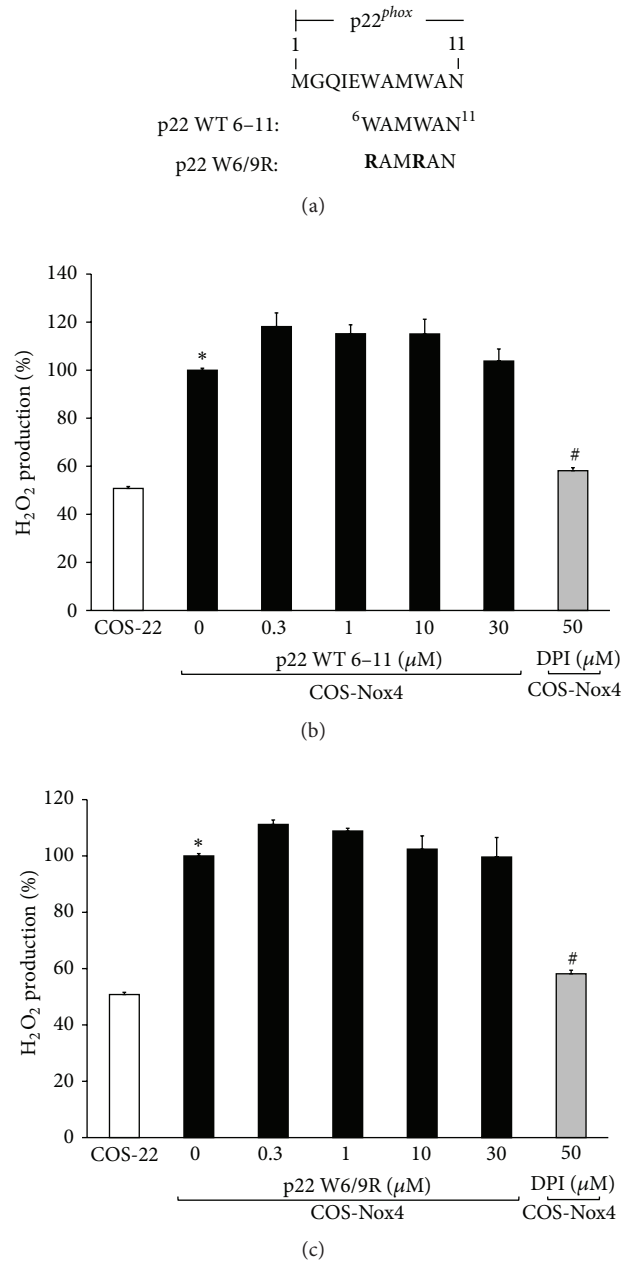


Figure 3: Testing whether $p22^{phox}$ N-terminal tail peptides inhibit Nox4 activity. (a) The first 11 amino acids of $p22^{phox}$ are displayed. The peptide sequence of $p22^{phox}$ between amino acids 6 and 11 is shown (p22 WT 6–11; WAMWAN); point mutations of W6/9R (p22 W6/9R; **R**AM**R**AN) are indicated in boldface type. The numbers at the N- and C-terminus of WAMWAN indicate the location of the corresponding residues in the amino acid sequence of $p22^{phox}$. (b) COS-Nox4 cell lysates were preincubated with various concentrations of p22 WT 6–11 for 15 min at 24°C, and H_2O_2 was measured using Amplex Red. Data represent the mean ± SEM of 3 experiments. (c) COS-Nox4 cell lysates were preincubated with various concentrations of p22 W6/9R for 15 min at 24°C, and H_2O_2 was measured using Amplex Red. Data represent the mean ± SEM of 3 experiments. $^*P < 0.05$ indicates a significant difference between COS-22 and COS-Nox4 cell lysates. $^\#P < 0.05$ indicates a significant difference between COS-Nox4 + 0 μM peptide and COS-Nox4 + DPI.

for this work was our previous findings illustrating that mimicking the corresponding B-loop region in Nox2, known as Nox2ds, selectively and potently inhibits Nox2-derived $O_2^{\bullet-}$ production [8]. We went on to provide evidence that Nox2ds, but not its scrambled control, acts as a competitive inhibitor of the enzyme. The theory then for this study was that these B-loop peptides would likewise act as competitive inhibitors of previously proposed key intramolecular interactions occurring between domains of Nox4. Surprisingly, peptide B90–98 up to 100 μM did not inhibit Nox4-derived H_2O_2 production as measured by Amplex Red fluorescence. Incidentally, Amplex Red was chosen as the most logical method for detecting H_2O_2, which is widely considered the primary Nox4 product [44].

Next, in the interest of pursuing a comprehensive approach to these studies, we progressed to testing whether peptides derived from the conserved regions of Nox4 B-loop were capable of inhibiting Nox4. We found that incubation of COS-Nox4 cell lysates with mimics of the N- and C-terminal conserved B-loop regions (B77–91, B82–96, and B92–106)

did not inhibit activity. With these results, we postulated that a B-loop peptide mimic might instead be replicating a normal intrinsic function of the B-loop. For this reason, we tested whether peptide B90–98 mutated at residue 96 (R96E), which previously was identified to be critical for Nox4 activity [32], inhibits Nox4-derived H_2O_2 production. We postulated that this mutated peptide could act as a dominant negative in that regard. Similar to the wild-type peptide, the mutant peptide did not inhibit Nox4-derived H_2O_2 production. One possible explanation for the lack of effect could have been that a multidimensional intramolecular interaction of the enzyme is at play, and thus targeting only one region could be insufficient to interfere with enzymatic activity. A second possibility is that Nox4 exists in a tightly assembled conformation and its unique tertiary structure permits an active electron transferring arrangement that cannot be disrupted by targeting discrete binding sites with either the native or mutant peptide.

A previous study demonstrated that a C-terminal region downstream of the NADPH-binding motif is important for Nox4, but not Nox2, activity [38]. With further analysis, von Löhneysen et al. identified the last 22 amino acids of Nox4 as essential to activity of the isozyme. Based on these data, we postulated that small peptides targeting this more flexible region would inhibit Nox4 activity. To test the hypothesis, sequential octameric and nonameric peptides were synthesized to encompass the last 22 amino acids of Nox4 and tested for their ability to inhibit Nox4. Likewise, our data demonstrate that none of these C-terminal tail peptides inhibited Nox4 activity. Taken together, these data appear to suggest that Nox4 exists in a tightly assembled, active conformation, thus explaining why peptides targeting intramolecular interactions of the enzyme are not able to interfere with its activity. This observation would be consistent with the reported constitutive and high capacity Nox4 activity.

Shifting to a new approach, we tested whether small peptides targeting the Nox4-$p22^{phox}$ intermolecular interaction could inhibit Nox4. A previous study showed that a peptide (175–194) derived from $p22^{phox}$ inhibits ROS production by Nox2 [42]. Subsequent studies by Dahan et al. using peptide walking identified domains throughout the $p22^{phox}$ protein sequence (9–23, 31–45, 47–61, 85–99, and 113–127) that are important for Nox2 activity [45]. It is, however, completely unknown whether introduction of $p22^{phox}$ peptides can disrupt Nox4-$p22^{phox}$ interaction and inhibit Nox4-derived H_2O_2 production. Importantly, a large part of the $p22^{phox}$ C-terminus does not seem to be important for Nox4 activity as deletion of amino acids up to and including the last 130 amino acids does not affect Nox4 activity [33]. Thus, targeting the C-terminal domain of $p22^{phox}$ is not likely to inhibit Nox4 activity. In contrast, deletion of the first 11, but not the first 5, amino acids at the $p22^{phox}$ N-terminus reportedly abolished Nox4-derived H_2O_2 production [33]. This suggested to us that the N-terminal region of $p22^{phox}$ between amino acids 6 and 11 might be sensitive to intervention. We targeted this region using a peptide sequence between amino acids 6 and 11 (p22 WT 6–11 (WAMWAN)) and measured Nox4-derived H_2O_2 production. Once again, the data demonstrated that p22 WT 6–11 did not inhibit Nox4 activity. We next considered previous work showing that mutation of tryptophans within this region to charged residues, such as arginine, abolished Nox4 activity [33]. Thus, in an attempt to mimic this inactive catalytic site, we tested whether the W6/9R mutant version of p22 6-11 (p22 W6/9R) could inhibit Nox4 activity. Similar to the wild-type peptide, the mutant peptide did not inhibit Nox4-derived H_2O_2 production.

As we observed, preincubation of COS-Nox4 cell lysates with Nox4 B-loop, Nox4 C-terminal tail, and N-terminal $p22^{phox}$ peptides for 15 min at 24°C did not inhibit H_2O_2 production. To allow more time for the peptides to disrupt the targeted domain interactions, the incubation time was increased up to 60 min. Notably, previous studies illustrate that 60 min is more than sufficient to inhibit Nox activity [40]. In our hands, 60 min incubation did not reveal inhibitory activity. To go one step further, these experiments were performed at 37°C in an attempt to increase access and likelihood of interaction of peptides with the Nox4 complex. Again, no inhibition of Nox4 was achieved using these peptides. Our data suggest that alternative strategies to improve access or penetrability of peptides into the Nox4-$p22^{phox}$ complex may be necessary to achieve inhibition of Nox4.

In conclusion, our findings suggest that the Nox4-$p22^{phox}$ complex is unperturbed by a wide array of rationally selected peptide mimics. This is not to say that other to-date unidentified active regions of the enzyme could not eventually be devised as inhibitors. Moreover, a more comprehensive study using peptide walking of the entire Nox4 protein could be warranted. It is also plausible that various combinations of peptide mimics may be effective. This is currently an area of active investigation in our laboratory. That notwithstanding, it is our tentative conclusion that the tightly assembled Nox4 complex creates a formidable barrier to peptidic interference and thus greater access could be viewed as the *sine qua non* of these strategies. In that vein, we are actively pursuing other strategies to temporarily improve access (unfolding) or penetrability of peptides into the Nox4 complex. Application of peptidic inhibitors targeting domain interactions of Nox4 while in the endoplasmic reticulum (before its native folding is complete or associates with $p22^{phox}$) may be another viable strategy. Of course, small molecule inhibitors that specifically target the above-identified interactions are likely to elude limitations of access and are currently a focus of intense interest in our laboratory.

Conflict of Interests

The authors declare that they have no conflict of intrests.

Acknowledgments

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Review Article

Inflammation and Hypertension: Are There Regional Differences?

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Hypertension is a chronic disease with global prevalence and incidence rapidly increasing in low and medium income countries. The surveillance of cardiovascular risk factors, such as hypertension, is a global health priority in order to estimate the burden and trends, to appropriately direct resources, and to measure the effect of interventions. We propose here that the adoption of Western lifestyles in low and middle incomes countries has dramatically increased the prevalence of abdominal obesity, which is the main source of proinflammatory cytokines, and that the vascular systemic inflammation produced by adipose tissue contributes to the development of hypertension. The concentration of proinflammatory cytokines is higher in the Latin American population than that reported in developed countries, suggesting a higher susceptibility to develop systemic low-degree inflammation at a given level of abdominal obesity. These particularities are important to be considered when planning resources for health care programs. Moreover, studying these singularities may provide a better understanding of the causes of the burden of cardiovascular risk factors and the remarkable variability in the prevalence of these medical conditions within and between countries.

1. Introduction

Hypertension is a chronic disease with global prevalence and rapidly increasing incidence in low and medium income countries, particularly in urban areas [1]. Indeed, while the prevalence of hypertension is decreasing in high income countries, it appears to be rising at alarming rates in low and middle income countries [2] where approximately 639 million people are living with hypertension. Moreover, in these countries a substantial proportion of hypertension is poorly controlled due to limited access to health services [1–7]. Clearly, the surveillance of cardiovascular disease risk factors such as hypertension is a global health priority, to estimate the burden and trends, to appropriately direct resources, and to measure the effect of interventions [2].

2. The Role of Hypertension in Cardiovascular Diseases

Considering that the main causes of mortality due to cardiovascular diseases (CVDs) include complications associated with hypertension, the lower awareness of this disease in low income countries is of great concern. Two recent large epidemiological studies that included low to middle income countries in Latin America, Asia, and Africa were conducted to determine the risk factors associated with a first myocardial infarction (the INTERHEART study [8]) and with a first stroke (the INTERSTROKE study [9]). These studies determined both global and region-specific population-attributable risk (PAR) for each major risk factor. Hypertension was identified as one of the primary risk

Table 1: Regional differences in the risk of stroke associated with hypertension (Adapted from: "O'Donnell, et al. [9]").

Region	Risk of stroke associated with self-reported hypertension or blood pressure > 160/90 mm Hg (Odds-ratio)
Africa ($n = 323$)^	4.96 (3.11–7.91)
Southeast Asia ($n = 1146$)‡	4.49 (3.54–5.70)
India ($n = 958$)	4.36 (3.34–5.69)
South America ($n = 151$)†	3.52 (1.63–7.60)
High income countries ($n = 422$)*	2.79 (1.83–4.25)

Data are odds ratio (99% CI). ^Mozambique, Nigeria, South Africa, Sudan, and Uganda. ‡China, Malaysia, and Philippines. †Argentina, Brazil, Chile, Colombia, Ecuador, and Peru. *Australia, Canada, Croatia, Denmark, Germany, Iran, and Poland.

factors for both outcomes, but it was also observed that particularly for stroke, the PAR associated with hypertension was substantially higher in low and middle income than in high income countries (Table 1).

It has been demonstrated in various animal models and from data in human twin and family studies that blood pressure is regulated by different genes [10, 11]. Nonetheless, many environmental risk factors are also associated with the development of hypertension. Factors associated with industrialization and urbanization such as obesity, high dietary salt intake, excessive alcohol consumption, social stress, and the ageing of the population are recognized as important contributory factors to the increases in blood pressure [5]. In low to middle income countries changes such as the increased access to westernized diets and the discontinuation of traditional dietary habits may have facilitated the expression of these pathologies and underlie the dramatic increases in the prevalence of hypertension observed in recent years [5].

3. The Role of Inflammation in Hypertension

Some years ago we demonstrated [12] that ultrasensitive C reactive protein (uCRP), a marker of low grade inflammation, was increased in individuals with hypertension. Based on these findings we hypothesized that low-degree inflammation could be an independent risk factor for essential hypertension [12], a proposal that has recently been reviewed and supported [13, 14].

As is shown in Figure 1, the process of urbanization and the adoption of Western lifestyles in low and middle income countries may play a role in the rise of abdominal obesity (AO) that has been described in these countries. Visceral fat is a relevant source of proinflammatory cytokines [15–17] which are significantly elevated in the serum of obese subjects [18]. It has been proposed that the vascular systemic inflammation produced by adipose tissue contributes to the development of hypertension, since inflammation produces endothelial dysfunction [19]. C-reactive protein (CRP), produced by the liver in response to the stimulus of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), is increased in subjects with multiple acute coronary events and is a strong independent predictor of new acute coronary events [20–22]. Moreover, we have demonstrated that in Andean women, CRP is an independent risk factor for pregnancy-induced hypertension [23, 24] and that in this population the concentration of CRP is increased in dyslipidemic subjects with MS [25, 26] as well as in overweight children [27].

Despite the differences in quantification methods and some disputed results, it appears that in general higher levels of proinflammatory cytokines are reported in non-Caucasians compared to Caucasians within the USA and UK (Table 2). These data support the proposal that there are ethnic differences in inflammatory markers that may also contribute to the variations in disease prevalence reported.

Recently, we conducted a study in schoolchildren in Bucaramanga, Colombia. The findings demonstrated a positive correlation between BMI, systolic blood pressure, and CRP [27]. These findings suggest that the correlation between CRP and hypertension previously reported in Colombian adults [12, 25] is also present among children and reinforces the idea that there is a link between adiposity (particularly visceral adiposity), increased BP, and increased plasma concentrations of inflammatory markers such as CRP. Several studies have reported ethnic differences in CRP and other proinflammatory cytokines concentrations in schoolchildren of developed and undeveloped countries (Table 3). Cook and colleagues [28] measured CRP concentrations in a representative sample of the population of England and Wales that included 699 children aged 10 to 11 years. That study showed that serum concentrations of CRP correlated positively with BMI, heart rate, systolic blood pressure, fibrinogen, and high-density lipoproteins, but not with other lipid fractions. Interestingly, it was found that the small number of children of South Asian origin had CRP levels 2.04 times higher than those from age, sex, and BMI-matched children. Ford [29] analyzed the results of the National Health and Nutrition Examination Survey of the United States (NHANES, 1999-2000), which included 2486 boys and girls aged between 3 and 17 years. BMI was the best predictor of CRP plasma concentration but the study also found ethnicity to be a determinant in boys aged 8 to 17 years and girls aged 8 to 11 years. Specifically, there was a higher CRP concentration in Mexican-American children in comparison with Caucasian-American children. In Colombia, we also found a significant positive correlation between BMI and CRP, and in accordance with higher levels of CRP in boys and girls from the upper tertile of BMI [27]. It is important to note that the CRP concentrations of the second tertile of BMI in Colombian children were as high as those reported in overweight and obese Caucasian-American and European children in a similar age range [29, 30].

On the basis of data from our study in children and previous studies in our adult population [12, 25, 26], it is interesting to propose that populations of low and middle income countries are predisposed to produce an inflammatory response at lower body fat levels than Caucasian populations. We suggest that the above is a consequence of a shorter time of

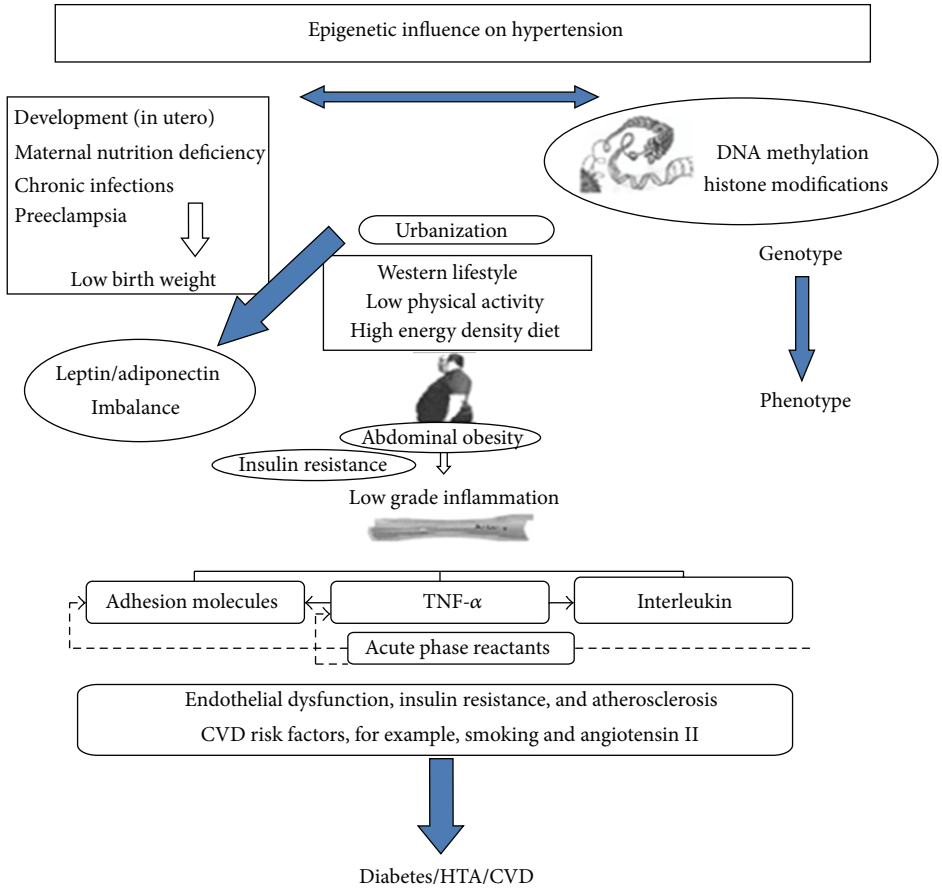

Figure 1: Early programming inducing stressors lead to alterations in gene expression (such as methylation of DNA or modification of histones) on phenotype producing persistent influences on metabolism. The conflict between the earlier programming and the later presence of abdominal obesity may have produced a higher sensitivity of this population to develop a state of low degree inflammation, insulin resistance and, consequently, an epidemic of hypertension, diabetes, and CVD.

exposure to the new lifestyles associated with modernization in these populations. We also propose that less exposure time leads to a delay in the adaptation process which results in a greater risk of low grade inflammation and insulin resistance at lower levels of abdominal obesity. Currently, poor hygiene (lack of potable water, defective waste, and sewage removal), high intestinal parasitism, infections, and tropical diseases are still present in Latin America. Superimposed on this proinflammatory background is the recent imposition of lifestyle habits that include diets high in saturated fats and refined flours, more smoking, increased physical inactivity, and abdominal obesity, all also associated with low grade inflammation [31, 32].

4. Why Are the Populations of Low and Middle Income Countries More Prone to Develop Low Grade Inflammation?

We suggest that this is a result of shorter exposure times among populations of developing countries to the new lifestyles associated with modernization. The shorter the exposure time, the less adapted the population is and the greater the risk of low-degree inflammation and insulin resistance at lower levels of abdominal obesity. Although the relative contribution of genetic, epigenetic, and environmental factors is not known, it is well documented that the Hispanic population in the USA and the South Asian population in the UK are at greater risk of low-grade chronic inflammation, DM2, and cardiovascular mortality than the Caucasian populations in those countries [33]. We have speculated that for minority populations who have recently migrated to high income countries from developing countries, the substantially shorter time of exposure to Western lifestyles is itself a risk factor for low grade inflammation. Data from the Pima Indians both exemplifies the influence of exposure to the Western lifestyle and demonstrates the greater susceptibility to these outcomes within indigenous populations with more recent exposure to it. The prevalence of both obesity and diabetes in the US Pima is substantially higher than in both their genetically similar counterparts living in Mexico and Caucasian Americans. In addition to genetic and environmental factors, intrauterine conditions

Table 2: Ethnic differences in main pro-inflammatory cytokines.

Study (Author and Ref)	Subjects	Marker	Results	Comments
Albert et al. [63]	24,455 White, Hispanic, and Asian adult females. Health Study in the United States.	CRP	Median/interquartile range (IQR) Black: 2.96 mg/L (1.19, 5.86) White: 2.02 mg/L (0.81, 4.37) Hispanic: (2.06 mg/L (0.88, 4.88) Asian: (1.12 mg/L (0.48, 2.25)	Black women had significantly higher values of CRP than White, Hispanic, and Asian. $P < 0.001$
Chandalia et al. [64]	137 South Asian and White adult males	CRP	Mean: Asian Indians: 0.99 mg/dL White: 0.58 mg/dL	Asian Indians had significantly higher concentrations of hs-CRP than Caucasians. $P = 0.036$.
McDade et al. [65]	229 Black, White, and Hispanic adult.	CRP	Median/(IQR) White: Females: 1.05 mg/L (0.44, 1.88) Males: 0.59 (0.44, 1.50) Black: Females: 3.30 mg/L (1.39, 4.47) Males: 1.07 mg/L (0.37, 1.70) Hispanic: Females: 1.49 mg/L (0.78, 3.10) Males: 1.00 mg/L (0.55, 1.65)	Black had significantly higher CRP concentrations than the other groups. $P = 0.007$
Schutte et al. [66]	217 Black and White adult females. POWIRS study.	CRP	Mean ± SD Black: 4.59 mg/L (3.17; 6.01) White: 3.27 mg/L (2.56; 3.98)	Black women had significantly higher hs-CRP levels compared to white women. $P < 0.05$
Patel et al. [67]	1083 Black and White adults. Bogalusa Heart Study.	CRP	Mean ± SD White: Males: 1.8 ± 1.9 mg/L; Females: 2.5 ± 2.3 mg/L; Black: Males: 2.3 ± 2.3 mg/L Females: 2.7 ± 2.4 mg/L	Black had significantly higher CRP values than Whites. $P < 0.01$.
Khera et al. [68]	2,749 White and Black adults. Dallas Heart Study.	CRP	Median Black: 3.0 mg/L White: 2.3 mg/L;	Significantly higher CRP values in blacks. $P < 0.001$.
Ford et al. [69]	2205 Whites, Black, and Mexican American adult females. National Health and Nutrition Examination Survey 1999-2000.	CRP	Mean Whites: 2.3 mg/L Black: 3.1 mg/L Mexican American: 3.5 mg/L	Significantly higher CRP in Mexican-American women than White women. $P < 0.001$.
Ford et al. [70]	1940 White, Black, Mexican, and other American adults.	CRP	Median: White: 1.6 mg/L Black: 1.7 mg/L Mexican: 1.5 mg/L Other: 1.8 mg/L	No significant differences between ethnicities.
LaMonte et al. [71]	135 Black, Native and White adult females. American Cross-Cultural Activity Participation Study (CAPS).	CRP	Mean ± SD Native: 0.25 ± 0.03 mg/dL Whites: 0.23 ± 0.13 mg/dL Black: 0.43 ± 0.03 mg/dL	Significantly higher CRP concentrations among Black compared with Native and White. $P = 0.002$.
Elkind et al. [72]	279 Hispanic, Black, and White American adult. Northern Manhattan Stroke Study.	CRP TNF-α IL-6 IL-1	Mean ± SD White: 1.88 ± 2.75 mg/L Black: 2.64 ± 4.62 mg/L Hispanic: 2.11 ± 3.50 mg/L White: 2.71 ± 4.25 pg/mL Black: 1.04 ± 1.63 pg/mL White: 1.15 ± 1.08 pg/mL Black: 1.36 ± 1.51 pg/mL White: 0.23 ± 0.43 pg/mL Black: 0.35 ± 0.59 pg/mL;	There were some differences in levels of marker by ethnicity but none were statistically significant.
Wener et al. [73]	22,000 multiethnic individuals age ≥ 4 yrs. Third National Health and Nutrition Evaluation Survey (NHANES III).	CRP	95th percentile value Males: 0.95 mg/dL Females: 1.39 mg/dL.	The values for Mexican-Americans and non-Hispanic whites were similar, compared with non-Hispanic black adults females, who had higher levels.

Table 2: Continued.

Study (Author and Ref)	Subjects	Marker	Results	Comments
Chatha et al. [74]	191 White and Indo-Asian. British adults.	CRP	Mean ± SD Indo Asian: Female 2.29 (1.52) mg/L Male 1.77 (1.46) mg/ L Whites: Female 2.23 (1.54) mg/ L; Male 1.94 (1.45) mg/ L.	Serum CRP concentrations were similar in Indo-Asians and White.
Chambers et al. [75]	1532 Asians and White. British adults.	CRP	Mean ± SD: Whites: 1.47 ± 1.62 mg/L Asians: 1.71 ± 1.81 mg/L	Significantly higher CRP concentration in Asians compared with whites. $P = 0.02$.
Forouhi et al. [76]	113 adult South Asian and White British adults.	CRP	Mean White: Male: 0.92 (0.34–1.61) mg/L Female: 0.70 (0.41–1.70) mg/L South Asian: Male: 1.07 (0.76–1.50) mg/L Female: 1.35 (0.72–3.04) mg/L	Median CRP level in South Asian women was nearly double that in European women. ($P = 0.05$).
		CRP	Mean Black: 2.5 mg/L Whites: 2.1 mg/L	Afro-Caribbean had significantly higher TNF-α ($P = 0.001$), and IL-6 ($P = 0.036$) levels.
Kalra et al. [77]	160 Black and White. British adults.	IL-6	Whites: 1.5 pg/mL Black: 2.3 pg/mL	No significance in CRP levels despite elevated IL-6 and TNF-α.
		TNF-α	Whites: 4.3 ± 3.6 mg/m/L Black: 6.7 ± 6.1 pg/mL.	CRP was significantly lower in Black men and women than in other ethnic groups. $P < 0.05$.
Heald et al. [78]	440 White, Pakistani, and Black British adults. Population-based community survey.	CRP	Mean Black: Male: 1.0 mg/L Female: 1.3 mg/L White: Male: 2.2 mg/L Female: 2.1 mg/L Pakistani: Male: 1.7 mg/L Female: 2.8 mg/L 2.8(2.1–3.6) mg/L	
Mwantembe et al. [79]	72 Black and White adults. Study performed in South Africa.	IL-1	Mean ± SD Whites: 1.99 ± 1.88 pg/mL Blacks: 2.69 ± 2.58 pg/mL;	No significant differences
Petersen et al. [80]	482 South-Asians and White young adults.	IL-6 TNF- α	Mean: Whites: 0.78 pg/mL South-Asians: 1.60 pg/mL; Whites: 1.13 pg/mL South-Asians: 1.29 pg/mL	Significantly higher IL-6 concentrations in South-Asians compared with White men. $P < 0.001$.
Albandar et al. [81]	228 White, Hispanic, Black adults.	IL-1	Mean: White: 28.4 pg/mL Hispanic: 34.7 pg/mL Black: 21.7 pg/mL	Hispanics had higher IL-1beta concentrations than Blacks. $P = 0.05$.
Hong et al. [82]	70 White, Black American Adults.	IL-6	Mean: IL-6 1.36 (±0.80) pg/mL.	No significant differences between ethnicities.

and "epigenetic" influences are also thought to contribute to the elevated risk of obesity in the US Pima [34]. Nonetheless, further research is needed to specifically evaluate our hypothesis that the *length* of exposure to the "obesogenic" Western lifestyle modifies the association between obesity and inflammation.

5. The Role of Environment and Epigenetics in Hypertension

Therefore, the dramatic increase in incidence of hypertension in low and middle income countries may be associated with rapidly changing environmental conditions interacting

Table 3: Pro-inflammatory cytokines in children and adolescents.

Study (Author and Ref)	Subjects	Marker	Results	Comments
López-Jaramillo, et al. [27]	325 schoolchildren (mean age, 10.0 years) from Colombia	CRP	Mean (mg/dL) ± SD Boys: 1.2 ± 2.6 Girls: 1.5 ± 2.0 BMI: 15 0.6 ± 0.9 BMI: 17 1.1 ± 2.2 BMI: 21 1.9 ± 3.7	CRP levels correlate significantly with BMI. ($P < 0.01$).
Gillum [83]	996 Mexican American children aged 6–11 years.	CRP	Detectable CRP was seen in 34.7% of overweight children but only 6.8% of other children ($P = 0.0006$, RR = 5.12, 95% CI: 3.32–7.90).	CRP levels correlate significantly with BMI. ($P < 0.01$).
Visser et al. [84]	3512 American children (8 to 16 years of age).	CRP	Percentile value CRP (mg/dL) CRP (4–11 years of age: >0.37 mg/dL for boys >0.68 mg/dL for girls Based on the BMI For overweight girls: 5.59 (95% CI: 2.20–14.22) For overweight boys: 6.12 (95% CI: 1.23–30.52)	CRP levels correlate significantly with BMI. ($P < 0.01$).
Cook et al. [28]	699 (10 to 11 years of age) multiethnic study in children.	CRP	Median mg/L All groups: 0.15 (IQ 0.06–0.47) South Asian: 2.40 (1.42, 4.04) Other: 0.82 (0.35, 1.87)	CRP was strongly related to adiposity (95% CI, 155–439%) and was higher in South Asian children.
Ford [29]	3348 White, Black and Mexican-American US children and young adults. National Health and Nutrition Examination Survey, 1999-2000,	CRP	Median White: 1.6 mg/L Black: 1.7 mg/L Mexican-American: 1.5 mg/L Other: 1.8 mg/L	No significant differences between ethnicities.
Aeberli et al. [30]	33 Swiss children (6 to 14 years of age). Normal-weight ($n = 33$), overweight ($n = 19$), and obese ($n = 27$)	CRP	CRP median (mg/dL) IL-6 (pg/mL) TNF-α (pg/mL) Overweight: 0.03 (0.01–0.42) Obese: 0.10 (0.03–0.23)	CRP, IL-6 increased significantly ($P < 0.02$) with increasing adiposity, independent of age.
		IL-6	Overweight: 0.34 (0.05–1.81) Obese: 0.41 (0.14–2.00)	
		TNF-α	Overweight: 6.3 (4.2–11.8) Obese: 7.2 (4.1–21.8)	

Table 3: Continued.

Study (Author and Ref)	Subjects	Marker	Results	Comments
Weiss et al. [85]	439 White, Black and Hispanic, obese, overweight and nonobese American children and adolescents.	CRP	Mean CRP (mg/dL), IL-6 (pg/mL) Blacks moderately obese: 0.13 Severely obese: 0.32 Whites moderately obese: 0.12 Severely obese: 0.31 Hispanics moderately obese: 0.13 Severely obese: 0.35	Interleukin-6 and CRP were significantly related to the degree of obesity ($P < 0.001$)
		IL-6	Blacks moderately obese: 1.89 Severely obese: 2.36 Whites moderately obese: 1.59 Severely obese: 1.80 Hispanics moderately obese: 2.07 Severely obese: 3.09	
Vikram et al. [86]	62 Indian adolescents	CRP	Mean (mg/dL) ± SD Normal weight: 2.5 ± 2.7 Overweight: 4.1 ± 2.4	CRP levels correlate significantly with BMI ($P < 0.05$).

with ethnic characteristics [14, 35]. Genetic predisposition associated with particular ethnic groups and lifestyle factors may also interact with in utero and early life conditions with respect to disease incidence. The *Developmental Origins of Disease* hypothesis emphasizes that there are critical periods in early life during which body structure and physiologic function are programmed for life. More recently, these effects of environment have been conceived in terms of epigenetics [35].

Epigenetics refers to functional alterations in gene expression or phenotype that do not change the underlying DNA sequence. These alterations induced by environmental conditions and mediated by modifications such as methylation of DNA or modification of histones can be transmitted to daughter cells thereby producing not only persistent, but also intergenerational influences on metabolism [36].

The mechanisms that control epigenetic processes are not completely understood, but it is clear that heritable DNA variation might alter the sensitivity to certain environmental triggers or change the nature of the epigenetic responses to a given exposure. In the Latin American context, the question is do regional and ethnic variations in epigenetic processes or simply differences in the environmental conditions explain the increased prevalence of hypertension?

Despite the increased prevalence of childhood and adult obesity in Latin America [32], maternal and childhood undernutrition remains a substantial public health problem within the region [32, 37]. While in children cardiovascular risk factors are strongly associated with BMI, somewhat paradoxically, a high prevalence of arterial hypertension is reported in stunted children and adolescents and adults within Latin America [38–41]. One study in Brazil [38] that investigated blood pressure in a random sample of adolescents who lived in slums and were exposed to nutritional stunting (10–16 years old, $n = 56$) showed that 51% had increased blood pressure and were at risk for hypertension. The prevalence of diastolic hypertension was 21% (95% CI = 10%–32%). The prevalence of cases with a systolic or diastolic arterial pressure above the 90th percentile was 51% (95% CI = 37%–65%). Another study conducted in the northeast of Brazil [39] included 416 adult slum residents and found hypertension in 28.5% of the population (women = 38.5%; men = 18.4%). They also observed that the height was associated with blood pressure, and in obese women lower height was associated with increased risk of hypertension (OR 1.98 95% CI 1.2–2.9). Another recent survey [40] investigated the association between height and health outcomes in mothers and offspring and found that short maternal height was independently associated with obesity, abdominal obesity, and increased arterial pressure, abdominal adiposity and high systolic blood pressure. Furthermore, short maternal height was associated with a low birth weight offspring and stunting in children. Also in Brazil, Franco et al. [41] reported changes in the sympathoadrenal and renin-angiotensin systems in children born small for their gestational age. They investigated the plasma levels of angiotensin-converting enzyme (ACE), angiotensin, and catecholamine's in 8- to 13-year-old children to determine correlations between the plasma

levels and both birth weight and blood pressure. Circulating noradrenaline levels were significantly elevated in small for gestational age girls compared to girls born with a weight appropriate for their gestational age. In addition, angiotensin II and ACE activity were higher in small for gestational age boys. There was a significant association between the circulating levels of both angiotensin II and ACE and SBP. Another study in Brazil [42] showed that ACE activity is increased, together with an increase in systolic and diastolic pressure, in children with stunting independent of birth weight.

Although in Latin America the prevalence of type 2 diabetes mellitus in individuals that were undernourished in early life is not known, it is known that poor countries with an accelerated process of urbanization are particularly vulnerable and have been experiencing a considerable increase in diabetes prevalence [43]. Deleterious changes have been reported in glucose metabolism in Mexican children suffering from undernutrition in infancy. The above-mentioned study examined the effects of undernutrition in the first year of life on glucose tolerance and plasma insulin levels. These authors reported that early postnatal undernutrition was associated with an increased incidence of alterations in the adult life even after adjusting for differences in birth weight [43].

It is interesting to speculate that the increased rates of hypertension, metabolic syndrome, and type 2 diabetes mellitus, observed in low and middle income countries, could be the result of the discrepancy between the nutritional environmental during fetal and early life and the adult environment. This discrepancy causes a mismatch between the fetal programming of the subject and the adult circumstances created by the imposition of new life styles [44]. The conflict between the earlier programming and the later presence of abdominal obesity may have produced a higher sensitivity of this population to develop a state of low-degree inflammation, insulin resistance and, consequently, an epidemic of hypertension, metabolic syndrome, and diabetes. The relative roles played by genetic and environmental factors and the interaction between the two are still subjects of great debate and merit further research.

6. The Role of Angiotensin II and Adiponectin in Hypertension

The visceral adipocytes of people experiencing the rapid changes described above are overexpressing the gene that regulates the synthesis of angiotensin II (Ang II) [45]. Ang II is produced in adipocytes [45–47], and it has been demonstrated that plasma levels of angiotensinogen and Ang II are increased with an increase in BMI [47]. Ang II has three important effects in humans, which were crucial to survival when human beings were nomads, fruit collectors, hunters, and fishermen and endured long periods without food. (1) It blocks insulin intracellular signaling routes, as a mechanism to conserve blood glucose [48]; (2) it stimulates the production of aldosterone, maintaining plasma sodium and water [49]; (3) it stimulates the production of proinflammatory cytokines, such as TNF-alpha, to maintain an alert state to fight infections [50, 51].

Nowadays, however, the production of Ang II in visceral adipocytes appears to be harmful and the insulin resistance and the water retention produced by Ang II are associated with hypertension, especially in low and medium income countries where the excess of fast food and sedentary lifestyles are relatively recent [35]. Moreover, it appears that the adaptation to this situation in obese people of developed countries, which have had a longer period of adaptation to the Western lifestyle, is an overexpression of adiponectin which in contrast to Ang II improves the insulin sensitivity and has anti-inflammatory effects [52]. This may explain why there is a substantially higher proportion of obese people who are metabolically healthy in high income countries compared to low and middle income populations. We propose therefore that the increased production of Ang II and the decreased production of adiponectin in visceral fat were an appropriate human biological response to the conditions of limited access to food and water. However, nowadays the imposition of Western lifestyles, which the humans in underdeveloped countries are not particularly well adapted to, is the main cause of the alterations that are leading to the increased prevalence of hypertension [35].

Using segments of internal mammary arteries obtained from adults with severe coronary artery disease (CAD), we showed [53] that the presence of obesity was associated with a higher contractile response to Ang II, after matching for age, sex, glucose and insulin plasma levels, homeostatic model assessment (HOMA) index, lipid profile, tobacco and alcohol consumption, physical activity, and arterial blood pressure. Moreover, increased waist circumference was associated with progressively lower levels of adiponectin and higher levels of leptin in these patients. We observed significantly higher concentrations of CRP and IL-6 in dyslipidemic patients with a history of CAD compared to those without a history of CAD [54]. Elevated levels of these inflammatory markers were not associated with any further impairment of endothelial function, but they were associated with a higher carotid intima-media thickness (IMT) in those subjects with a previous history of CAD. These results suggest that in our population low grade inflammation is associated both with adiposity and with the progression of CAD.

Ang II has been proposed as a trophic factor in white adipose tissue growth and development, since renin-angiotensin system components are influenced by nutritional state and adipose tissue mass [55–61]. Higher Ang II is also associated with lower birth weight [41]. Increased thermogenesis could also participate in the reduction of body weight, and Ang II seems to be also related to this effect [41]. The local renin-angiotensin system plays a role in adipocyte differentiation and in body-fat accumulation. In humans Ang II produced by mature adipocytes appears to inhibit the differentiation of adipocyte precursors, thus decreasing the percentage of small insulin-sensitive adipocytes and promoting the presence of large adipocytes [56–58], which decrease insulin sensitivity and produce ectopic deposition of lipids that promotes the development not only of hypertension, but also of insulin resistance and type 2 diabetes [62].

7. Conclusions

Hypertension, diabetes, and that cluster of metabolic alterations often referred to as the metabolic syndrome are highly prevalent in low and middle income countries which contribute to an increasing proportion of the worldwide burden of chronic disease. Ethnic differences in low grade inflammation are already evident in childhood and large epidemiological studies clearly show regional differences in the associations between AO, inflammation, and hypertension. While prevalence varies between countries within each region and within different areas in these countries [87], it appears that compared to developed countries populations within developing countries, have a greater susceptibility to hypertension and other cardiometabolic disease at a given level of adiposity. We argue that in low and middle income countries, elevated CRP (and other inflammatory markers) and angiotensin II, associated with the higher prevalence of maternal malnutrition and early growth restriction or childhood under nutrition, are important contributors to the higher susceptibility to hypertension and cardiovascular disease observed within these regions. In accordance, region-specific research is urgently needed to better understand interactions between genetic, epigenetic, and environmental factors operating in populations being rapidly exposed to Western lifestyles. Moreover, as recently we have showed, in our population nutritional intervention as a supplementation with aged garlic can increase the levels of adiponectin, probably contributing to improve the metabolic profile of Colombian subjects with abdominal obesity and hypertension [88].

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Review Article

Hypertension in Metabolic Syndrome: Vascular Pathophysiology

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Metabolic syndrome is a cluster of metabolic and cardiovascular symptoms: insulin resistance (IR), obesity, dyslipemia. Hypertension and vascular disorders are central to this syndrome. After a brief historical review, we discuss the role of sympathetic tone. Subsequently, we examine the link between endothelial dysfunction and IR. NO is involved in the insulin-elicited capillary vasodilatation. The insulin-signaling pathways causing NO release are different to the classical. There is a vasodilatory pathway with activation of NO synthase through Akt, and a vasoconstrictor pathway that involves the release of endothelin-1 via MAPK. IR is associated with an imbalance between both pathways in favour of the vasoconstrictor one. We also consider the link between hypertension and IR: the insulin hypothesis of hypertension. Next we discuss the importance of perivascular adipose tissue and the role of adipokines that possess vasoactive properties. Finally, animal models used in the study of vascular function of metabolic syndrome are reviewed. In particular, the Zucker fatty rat and the spontaneously hypertensive obese rat (SHROB). This one suffers macro- and microvascular malfunction due to a failure in the NO system and an abnormally high release of vasoconstrictor prostaglandins, all this alleviated with glitazones used for metabolic syndrome therapy.

1. Introduction

The metabolic syndrome is a cluster of metabolic and cardiovascular symptoms that are strongly associated with type II diabetes mellitus. In this kind of diabetes, rather than prolonged high levels of glycemia, there is insulin resistance with secondary hyperinsulinemia, both very frequently associated with, hypertension, dyslipemia, atherosclerosis, and, most importantly, obesity (Figure 1) [1]. Vascular disorders are central to this condition. Quoting prof. Yki-Järvinen "...after all, from a clinical point of view, type II diabetes mellitus is a disease of blood vessels, not muscle." [2]. For these reasons, it is also known as cardiometabolic syndrome [1], and hypertension plays a pivotal role. Indeed, risk estimates according to the Framingham study show that roughly 80% of essential hypertension in men and 65% in women can be directly attributed to obesity [3]. There is a clear association between body mass index and arterial pressure even in nonobese, lean people [4–6]. Still, some obese people are not hypertensive. For example, the North American Pima Indians, who have a high prevalence of obesity, but do not have corresponding high rates of hypertension [7].

The history of metabolic syndrome takes us back to the early 20th century, when two physicians, the Swedish, Kylin and the Spanish Marañón nearly simultaneously and independently published in the journal *Zentralblatt für Innere Medizin* two articles under almost the same title: *Über Hypertonie und Zuckerkrankheit* [8, 9]. In these articles, the two physicians described for the first time the coexistence of hypertension and diabetes mellitus in adults and proposed a common mechanism for the development of these disorders. In 1988, Reaven, hypothesized that insulin resistance is the common etiological factor of a group of disorders, such as high blood pressure, hyperinsulinemia, high levels of low density lipoproteins (LDL), triglycerides, and cholesterol, and low levels of high density lipoproteins (HDL). Reaven named this collection of disorders "syndrome X" [1]. A year later, Kaplan added to the pathologies described by Reaven a very important factor, central adiposity (increase in splanchnic and subcutaneous fat depots in the abdominal region) [10].

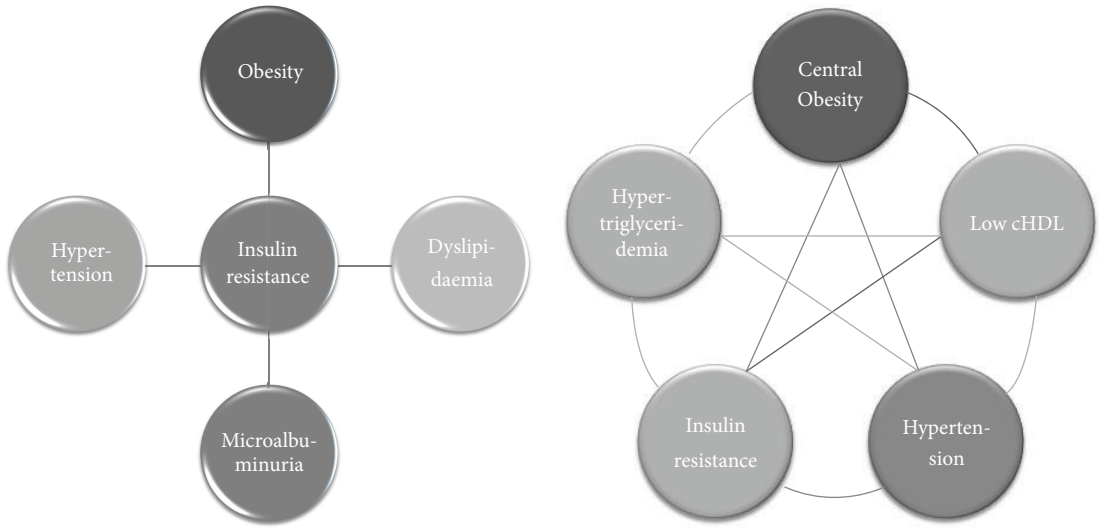

Figure 1: Two ways to conceptualize metabolic syndrome and the position hypertension and the other symptoms occupy. According to the WHO definition, insulin resistance is central to any other symptom (a). Others define metabolic syndrome as a cluster of symptoms where none has a central position (b).

Since then, abdominal obesity has been considered one of the typical components of the syndrome.

Both type 2 diabetes mellitus and metabolic syndrome are reaching epidemic proportions. Considering that 220 million people worldwide are diabetic, this disease has become a serious epidemiological problem [11]. The problem is not only the size of the figures but also the alarming increase in only a few decades (46% in the 1990s). Metabolic syndrome is, probably, the most important challenge for health authorities in developed and developing countries [11, 12]. In Europe there is a clear North-South gradient in almost all cardiovascular risk factors related with metabolic syndrome. For example, mortality from coronary heart disease, expressed as a mortality ratio, presented in men aged 30–69 the following geographical indices: 8.2 Iceland; 5.1 England; 2.2 Italy; 1.8 Spain; and 0.9 Portugal [13]. However, there is no doubt that the paradigm of overdevelopment-overweight is the United States. With the turn of the century, 61% of Americans were sufficiently overweight to suffer health problems directly derived from this condition [14]. A diet that is as excessive as inadequate has yielded these epidemiological figures in less than 20 years: between 1977 and 1995 daily caloric intake rose by 200 calories. This is the equivalent to an increment of 10 calories per year [14].

2. Role of the Sympathetic Nervous System

There are 3 conditions, typical of metabolic syndrome, that may cause an exacerbation of sympathetic tone. Namely, hyperinsulinemia, hyperleptinemia, and hyperlipidemia. In 1981, it was reported that hyperinsulinemia, independently of changes in glycemia, caused a substantial increase in circulating noradrenaline concentration accompanied by an increase in blood pressure [15]. These sympathoexcitatory effects of insulin appear to be centrally mediated, since they are apparent only during systemic insulin infusion but not local infusion [16]. In addition, high levels of insulin increase sodium reabsorption [17] favouring expansion of extracellular fluid volume, which may predispose to hypertension [18]. Furthermore, obesity impairs renal-pressure natriuresis and causes sodium retention. Obese subjects require increased arterial pressure to maintain sodium balance, indicating impaired renal-pressure natriuresis [19].

In addition to insulin, leptin can also be a link between obesity and increased sympathetic activity. Besides its effect on appetite and metabolism, leptin acts in the hypothalamus to increase blood pressure through activation of the sympathetic nervous system [20]. High circulating levels of leptin are reported to explain much of the increase in the renal sympathetic tone observed in obese human subjects [21]. Leptin-induced increases in renal sympathetic activity and blood pressure are mediated by the ventromedial and dorsomedial hypothalamus [22].

Finally, high circulating levels of free fatty acids in visceral obese individuals may participate in the activation of the sympathetic nervous system. The increased release of free fatty acids into the portal vein from lipolysis in visceral fat depots could explain the strong association between visceral obesity and increased sympathetic nerve outflow [23].

3. Role of Insulin

3.1. Insulin Resistance and Endothelial Dysfunction. In 1939, Himsworth postulated that type 2 diabetes mellitus was not only an insulin deficiency state but also a disease in which cells are unresponsive to insulin. Thus, Himsworth's work gave birth to the concept of insulin resistance [24, 25]. Insulin resistance is clinically defined as the inability of a known

quantity of insulin (exogenous or endogenous) to increase glucose uptake and utilization in an individual as much as it does in a normal population [26]. There is a clear link between endothelial dysfunction and insulin resistance [27, 28] but the mechanism by which insulin resistance leads to endothelial dysfunction is complex and involves the action of mediators of inflammation in the visceral fat, liver, and muscle [29]. It is well known that insulin resistance and compensatory hyperinsulinaemia, besides activating the mechanisms mentioned above, have also a vascular toxicity effect, mainly at the endothelial level. This, partly because insulin resistance impairs the production of NO, favors the production of endothelin-1 and the vasoconstrictive and mitogenic responses on the vascular wall [30].

3.1.1. Role of NO in Insulin Resistance. King and Johnson reported in 1985 that the endothelial cell membrane displays insulin receptors [31]. Functional studies indicate that endothelium-derived NO is involved in the insulin-elicited increase in blood flow and recruitment of capillaries that physiologically links hemodynamics to the metabolic action of insulin on the tissues [32–34]. Insulin resistance is associated with impaired NO synthase activity [35] and an abnormal basal NO-mediated dilation in the forearm arterial bed [36]. The insulin-induced increase of microvascular endothelium-dependent vasodilation is abolished in insulin resistance conditions such as obesity [37]. Moreover, insulin has been shown to constrict rather than dilate forearm resistance arteries in obese patients [38]. On the other hand, inhibition of NO synthesis or endothelium removal reveals a vasoconstrictor effect of insulin on isolated arterioles [39]. Definitive proof of the relationship between NO and insulin sensitivity has been provided by knock-out mice that are homozygous null for the eNOS gene. These peculiar animals display an expected hemodynamic phenotype of increased basal blood pressure but also are insulin resistant [40]. Therefore, insulin has indeed a hemodynamic component, albeit small compared to the metabolic one. But both are coupled in such a manner that endothelial dysfunction can cause insulin resistance, and this, in a vicious circle, aggravates endothelial function.

Interestingly, insulin-signaling pathways in vascular endothelium leading to the activation of endothelial NO synthase are completely independent and distinct from classical calcium-dependent mechanisms used by G-protein-coupled receptors, such as the acetylcholine receptor [34]. The messenger pathway that is activated when insulin binds insulin receptor appears to be as follows [41]: insulin binds insulin receptor (INS-R) which is at the same time a tyrosine kinase and this undergoes autophosphorylation of tyrosine residues. INS-R phosphorylates insulin receptor substrate-1 (IRS-1). The signalling pathway from insulin branches at IRS-1. One of the branches involves the activation of phosphoinositide 3 kinase (PI-3K), leading to phosphatidylinositol-3,4,5-triphosphate as well as to phosphorylation and activation of phosphoinositide-dependent kinase 1 (PDK-1). Both products, in turn, phosphorylate and activate Akt (also called protein kinase B, PKB). Akt directly phosphorylates eNOS at Ser^{1177}, resulting in increased eNOS activity and NO production [42]. Remarkably, the vascular actions of insulin that stimulate the production of NO possess remarkable similarities to metabolic insulin-signaling pathways. For instance, activation of Akt is also a common step for glycogen synthase kinase inhibition and GLUT-4 transporter translocation [41].

3.1.2. Role of Endothelin-1 in Insulin Resistance. In 1991, Oliver et al. demonstrated that insulin was able to stimulate endothelin-1 (ET-1, a very strong vasoconstrictor) gene expression in endothelial cells [43]. Later, it was shown that insulin can modulate circulating ET-1 levels [44] and increased plasma levels of ET-1 were observed in type II diabetic patients [45]. An additional work in the skeletal muscle circulation reported that insulin stimulates both NO activity (already known as we showed before) and ET-1 [46].

The authors then suggested that an imbalance between the release of both substances may be involved in pathophysiology of hypertension and atherosclerosis in insulin-resistant states associated with endothelial dysfunction [46]. Following research has shown that insulin induces endothelin-mediated vasoconstriction only when NO synthase or phosphatidylinositol-3 kinase (PI3K) is inhibited [47]. In a paper elegantly entitled "Endothelin antagonism uncovers insulin-mediated vasorelaxation in vitro and in vivo" [48], Verma et al. demonstrated that insulin-mediated vasorelaxation is only well patent when antagonizing ET-1 receptors. This proved previous proposals that insulin exhibits a dual and opposite action on blood vessels: NO-mediated vasodilation and ET-1-mediated vasoconstriction. It is known that MAPK activation by IRS-1 causes the release of endothelin-1, which promotes insulin resistance (by reducing blood supply to the skeletal muscle), increases oxidative stress, reduces the bioavailability of NO, and promotes a proatherogenic state [49].

3.2. Hyperglycaemia and Vascular Function. Regardless of the evidence linking the vascular dysfunction of type II diabetes mellitus with failures in the vascular biology of insulin, there are many reports that attribute these dysfunctions to the very fact of the existing hyperglycaemia. We wish to draw attention to the functional effects of the acute excess in glucose occurring in a particular moment. In this regard, it has been reported that glucose favours vasoconstriction [50] and impairs vasodilation [51]. In arteries of diabetic rats, Taylor et al. demonstrated that hyperglycaemia reduces the tonic release of NO [52] and established a central role for glucose in the development of vascular functional changes associated with experimental diabetes [50]. Most interesting is the finding that in healthy subjects, acute hyperglycaemia impairs endothelium-dependent vasodilation in both the microcirculation and the macrocirculation when assessed in the brachial artery [53]. More precise data on the mechanisms involved in hyperglycaemia was released by Sobrevia et al. [54] who showed that exposure of endothelial cells to elevated glucose was associated with stimulation of L-arginine transport paralleled by an increase in basal release of NO and prostacyclin. This would be good news if they

did not find as well that insulin treatment downregulated the elevated activity of the L-arginine transport system and that of NO synthase in the cells exposed to hyperglycaemia. They concluded that the modulation of the human endothelial cell L-arginine-NO pathway by insulin is influenced by predisposing hyperglycaemic clinical conditions [54]. In a later study, Renaudin et al. demonstrated that the vasodilatory effect of insulin disappears when hyperglycaemia exists, perhaps blunted by the vasoconstrictive effect of glucose [55].

3.3. Insulin Actions on Blood Pressure: The Insulin Hypothesis of Hypertension. So far we have focused on the cardiovascular effects of insulin at a local level. However, it cannot be forgotten that insulin has systemic actions affecting the sympathetic nervous system and kidney. The surge of epidemiological reports relating insulin resistance and hyperinsulinemia has fueled the idea of the so-called insulin hypothesis of hypertension. There is no question that insulin resistance is epidemiologically linked with hypertension [1]. The insulin hypothesis of hypertension proposes that the compensatory hyperinsulinemia that occurs with insulin resistance increases sodium reabsorption and sympathetic activity, which combine to cause elevated arterial pressure. Support for this hypothesis comes from various lines of evidence. First, the correlation between insulin resistance and high blood pressure [56], which is emphasized by the fact that, even lean individuals with essential hypertension, display insulin resistance and hyperinsulinemia. Some go a step further asserting that essential hypertension is "per se" an insulin resistance state [57]. Second, as explained before, insulin has multiple actions on the sympathetic nervous system, the kidney, and the vasculature which can lead to hypertension. Third, the observation that drugs which improve insulin resistance and decrease hyperinsulinemia, are reported to be antihypertensive. For instance, Landin et al. reported that oral administration of metformin to insulin-resistant, hypertensive men increased insulin sensitivity and significantly decreased arterial pressure [58]. Another remarkable example is the well-known blood pressure lowering effects of insulin sensitizers glitazones [59]. For review, see [60]. Fourth and finally, the observation that some antihypertensives, such as angiotensin II converting enzyme inhibitors [61] or angiotensin II receptor antagonists [62], increase insulin sensitivity as well. Despite the size of the support in favour of the insulin hypothesis of hypertension, there is also important evidence against. For instance, the eminent physiologist Hall and his collaborators failed to find a correlation between insulin and hypertension in a well-controlled model in dogs [63].

4. Role of Adipokines

Traditionally, adipocytes were considered energy reservoirs that store triglycerides during feeding and deliver fatty acids during fasting. However, it has become quite clear that adipose tissue does much more than this and is responsible for the synthesis and secretion of numerous proteins. The first protein described was adipsin [64]. Later, the secretion of cytokines such as TNF-α was described [65], thus conferring immune functions to adipocytes. Funahashi et al. named these substances adipocytokines [66]. Undoubtedly, the most relevant discovery was leptin by the Friedman group in 1994 [67]. Because the vast majority of substances produced by the adipocyte are not necessarily cytokines, Trayhurn and Wood recommended the term *adipokines* instead. Therefore, adipokines are defined as any substance synthesized and secreted by the adipocytes [68]. Thus, it has become quite clear that adipose tissue is indeed an endocrine organ. In fact, it can be the largest organ in the body. This is physiologically and pathophysiologically important because the total amount of secreted adipokines are enormous and may affect the whole body economy, especially considering that every adipocyte is connected to the vascular network [69]. It is well known that dysregulation of the production and secretion of adipokines is involved in the development of metabolic and cardiovascular diseases. In metabolic syndrome, intra-abdominal visceral fat accumulation has been shown to play a key role in the development of a variety of metabolic and circulatory disorders through the dysregulation of adipokine secretion [70].

4.1. Perivascular Adipose Tissue and Vascular Function. The function of adipose tissue as an endocrine organ has important implications in the understanding of the pathophysiological relationships between excess body fat and hypertension. Almost all the systemic arteries are surrounded by a layer of perivascular adipose tissue (PVAT). In the majority of myographic studies, PVAT is removed on a routine basis. This is a custom based on the assumption that PVAT can prevent the diffusion of vasoactive substances. This is perhaps the reason that, despite the ubiquity of PVAT, very little is known about its function in vascular biology. Perivascular fat certainly has a modulator action on vascular contractility. This was described by Soltis and Cassis in a study published in 1991 [71]. This work has often been misinterpreted as the first postulator of a supposed prorelaxing role of PVAT. These researchers describe a decrease in the sensitivity to noradrenaline when aortic segments remain with PVAT. They demonstrate that this is due to the uptake and elimination of this catecholamine by adipose tissue. They postulate that the nerve endings within PVAT recapt and remove noradrenaline within the synaptic gap. This obviously results in a buffered effect of this neurotransmitter, but it is not postulated that PVAT releases any anticontractile factor.

In more recent years, several groups have dealt with the possible vasoactive role of PVAT. The group of González et al. has been especially interested in the vasoactive properties of the tunica adventitia, to which they attribute a role in the contractile ability of the responses modulated by the endothelium [72]. Later on, Gao et al. as well as Rey et al. claimed that PVAT promotes the vasoconstrictor response to electrical stimulation [73] and impaired endothelial function [74] via reactive oxygen species generated by NADPH oxidase. On the other hand stands the work pursued by Gollasch's group and initiated by Löhn et al. who claim to have found a diffusible factor derived from PVAT, which

they called "adventitium-derived relaxing factor" or ADRF [75]. In a following paper, the "A" standed for "adipocyte" instead [76]. A relevant amount of literature has confirmed the existence of this anticontractile diffusible substance (see [77] for review). Still, there is no unanimity regarding the nature and mechanism of action of ADRF. For Verlohren and coworkers, it is independent from the endothelium [76], but not for Gao et al. [78]. What seems clear is that the vasodilatory effect of ADRF is mediated by the opening of different K^+ channels on vascular smooth muscle cells [75, 76, 78–80]. Endocrine and vascular paracrine functions of a variety of adipokines are shown in Table 1. We shall focus on those with particular vasoactive actions, namely, leptin, adiponectin, TNF-α, prostaglandins, angiotensin II, and endothelin-1.

4.2. Leptin. The discovery that the endothelium expresses the leptin receptor OB-Rb [81], converted endothelial cells, just like those of the hypothalamus, in a target for this hormone. The presence of leptin receptors in the vascular endothelium and not only in the central nervous system is important because it allows to find a link between leptin and altered vascular function in obesity [82]. Leptin is an NO-dependent vasodilator but also increases peripheral vascular resistance and sympathetic nerve activity [83]. The concentration of plasma leptin is correlated with adiposity, and hyperleptinemia is indeed considered an independent cardiovascular disease risk factor [84]. There are two theories that relate leptin's cardiovascular effects to obesity. One of them proposes that leptin is involved in the control of vascular tone simultaneously causing a neurogenic pressor action and an opposite depressor effect mediated by NO [85]. Another theory, based on experiments performed in coronary arterioles [86], proposes that, paradoxically, leptin causes itself NO-dependent vasodilation and, at the same time, its very presence impairs endothelium-dependent relaxations, that is, produces endothelial dysfunction. The problem with this interesting theory is that leptin-induced relaxation occurs at concentrations well above those found in very obese subjects. Physiological (lean) or pathophysiological concentrations (obese) of leptin have, however, little direct effect on vascular tone. Possibly, the most relevant aspect of this theory is that leptin concentrations actually existing in obese patients do elicit endothelial dysfunction [86].

4.3. Adiponectin. Adiponectin is the secretory protein produced in largest amounts by adipocytes and present in high and stable concentration in the plasma. In healthy subjects, adiponectin carries out its roles preventing the development of vascular changes and has been reported to be associated with lipid metabolism [87], glucose metabolism [88], and insulin resistance [89]. Unlike leptin, plasma adiponectin levels are negatively correlated with body mass index. This negative correlation is stronger between adiponectin levels and visceral adiposity than between the protein and subcutaneous adiposity [90]. Also, there is a close relationship between low concentrations of adiponectin in the blood, insulin resistance, and hyperinsulinemia. It has been suggested that the decrease in plasma adiponectin concentration contributes to the metabolic complications associated with obesity [91]. Adiponectin improves NO-dependent vasodilation by opening voltage-dependent potassium channels [92–94].

Some reports suggest that adiponectin plays an important role in insulin actions and hypoadiponectinemia may result in insulin resistance and diabetes mellitus. In fact, Lindsay et al. demonstrated that plasma levels of adiponectin were lower in Pima Indians, a unique cohort with high prevalence of obesity [95]. They also demonstrated that plasma levels of adiponectin are strongly correlated with insulin sensitivity evaluated by glucose disposal rate [96]. The study of the Pima Indian population demonstrates that adiponectin may play a crucial role in the development of diabetes mellitus and that high adiponectin levels should protect from the deterioration of glucose metabolism. Thus, hypoadiponectinemia could be a significant background of vascular changes and metabolic disorders, including insulin resistance and, possibly, a background for hypertension as well. Indeed, some studies show that hypertensive subjects have lower levels of plasma adiponectin [97].

4.4. Tumor Necrosis Factor-α (TNF-α). Since Hotamisligil's group reported that adipose tissue expresses TNF-α, one of the candidate molecules inducing insulin resistance adipokines [98], this factor has been recognized as one of the most important adipokine. Adipocytes secrete TNF-α, and the expression of this factor is increased in the hypertrophied adipocytes of obese subjects. TNF-α is the molecule linking inflammation with obesity [99]. We will further discuss this adipokine in the diet-induced hypertension section.

4.5. Prostaglandins (Adipocyte Derived). Prostaglandins, together with angiotensin II and endothelin-1, are the most vasoactive substances generated by adipocytes. Adipocytes produce prostaglandins in response to sympathetic stimulation. Lipolytic hormones, like adrenaline, are linked to the hypertensive status and obesity-associated hypertension. These hormones target membrane adipocyte β receptors and in turn activate hormone sensitive lipase. This stimulus induces lipolysis, release of fatty acids, and prostaglandins, especially PGE_2 and PGI_2, which are also fatty acids in origin. Antilipolytic stimuli, insulin, for example, reduce the release of prostaglandins [100] such as prostacyclin (PGI_2). On the basis that insulin decreases the production of this strong vasodilator, Parker and coworkers suggested that hypertension associated with insulin resistance and hyperinsulinemia (i.e., metabolic syndrome) would be due partly caused by the lack of proper PGI_2 release [69]. It appears that PGI_2 production by the adipocytes results from the cooperation of adipocytes and vascular endothelial cells. Parker and coworkers proved that adipocytes are a source of the original fatty acid component of prostaglandins, arachidonic acid, that is converted into prostaglandins by the closely located vascular endothelial cells. Adipocytes provide arachidonic acid but lack the required cyclooxygenase which

Table 1: Endocrine and vascular paracrine functions of some adipokines.

Adipokine	General effects	Vascular effects	References
Leptin	Satiating factor Physiological regulation of feeding behaviour through hypothalamic receptors Levels correlate with amount of body fat	Endothelial dysfunction Endothelium-dependent and independent relaxation	[67, 85, 160–165]
Resistin	Relates obesity to diabetes by inducing insulin resistance	Impairs endothelial function due to an increase in ET-1 production and a decrease in NO production	[166, 167]
Adiponectin	Levels inversely correlate with obesity	NO-dependent vasorelaxation mediated by K_v channels	[91, 93, 94, 168]
Visfatin	Expression correlates with obesity degree Similar effects to insulin in cell culture	NO-dependent vasorelaxation	[169–171]
TNFα	Links inflammation with obesity Increase in TNFα expression induces ROS production Reduces adiponectin production	Endothelium-dependent and -independent vasodilatation Triggers ET-1 and Ang II-induced vasoconstriction Impairs endothelium-dependent vasodilatation due to increased ROS production or decreased NO production Less vasodilatory effect of PAT due to ROS production	[94, 99, 172–178]
Interleukin-6	Contributes to systemic inflammation and insulin resistance	Endothelium-independent vasodilatation Endothelial dysfunction due to an increase in ROS production and decreased NO production	[94, 179–182]
Prostanoids	See vascular effects Hemostasis Numerous biological functions	Vasoconstriction or vasodilatation depending on which prostanoid	[183, 184]
Angiotensin II	See vascular effects Na^+ and water homeostasis Renal function	Vasoconstriction	[185, 186]
Endothelin-1	See vascular effects	Vasoconstriction	[187]
Reactive oxygen species	Numerous biological effects Ageing	Vasoconstriction through Ca^{2+} sensitization Decrease in NO bioavailability	[73, 188, 189]
Adventitial derived relaxing factor	See vascular effects	Vasorelaxation through opening different K^+ channels	[75, 76, 78–80]

is provided by adjacent endothelial cells [69]. However, adipocytes do express cyclooxygenase [101], and according to Richelsen et al., adipocytes can synthesize prostaglandins, but still provide endothelial cells with adipocyte-derived arachidonic acid to further generate prostaglandins [100].

4.6. Angiotensin II (Adipocyte Derived). The first to propose PVAT as a source of angiotensin II were our previously quoted Soltis and Cassis who suggested that adipocyte-derived angiotensin II would favor vasoconstriction [71]. This effect could be due to the fact that the angiotensin II action prevents PI3K activation, resulting in a loss of stimulation of NO synthesis by this route [102], as discussed in the section related to endothelin-1. Plasma renin activity and thus the production of angiotensin II are high in obese individuals [5, 19]. Three possible explanations have been proposed to explain this phenomenon: (1) obesity may raise renin secretion by increasing loop of Henle sodium chloride reabsorption and reduce sodium chloride delivery to the macula densa [19]; (2) obesity may stimulate renin secretion by activation of the sympathetic nervous system [19]. Finally, (3) the existence of a high renin activity in the hypertrophied adipocytes causing an increased angiotensin II release [103–106]. Today, we know that adipocytes possess the whole enzymatic machinery involved in the renin-angiotensin system [103] and, in fact, they do synthesize angiotensin II [105, 107]. Importantly, angiotensinogen gene expression is higher in intra-abdominal fat than in other fat depots or nonadipose tissues [108]. Indeed, increased production of angiotensinogen by intra-abdominal fat appears to explain the high circulating levels of this peptide observed in dietary obesity [104]. Closely related with the physiology of angiotensin II is aldosterone. The levels of this corticoid are elevated in some obese hypertensives, especially patients with visceral obesity [109]. Furthermore, it has been recently discovered that adipocytes also produce aldosterone (actually in response to angiotensin II) [106]. In this regard, the adipocyte may be considered a miniature renin-angiotensin-aldosterone system.

It is noteworthy that adipose cells also secrete mineralocorticoid-releasing factors with important effects on aldosterone release from adrenocortical cells [110]. These are called *adipogensins* or aldosterone-releasing factors (ARF) [111] but are not well characterized as yet. There is a lot of data that suggests a close relationship between an excess in released aldosterone and insulin resistance. Aldosterone promotes insulin

resistance through mineralocorticoid receptors activation (independently of gene transcription) in a large number of tissues [112]. On the other hand, hyperinsulinaemia induces increase in aldosterone levels [113, 114] thus creating another positive feedback cycle between hyperaldosteronism and hyperinsulinemia, with important pathophysiological effects in subjects with insulin resistance and a potential mechanism for the development of complications in obese hypertensive patients.

4.7. Endothelin-1 (Adipocyte Derived). As stated in previous lines, endothelin-1 is a vasoconstrictor protein normally produced by the endothelial cells but qualifies as adipokine as well [115]. Indeed, the levels of endothelin-1 increase in obesity and type II diabetes [116, 117]. In studies of experimental obesity, an increase in endothelin-1 gene and protein expression has been detected within the cardiovascular system [118]. Harmelen et al. found that obese adipose tissue releases 2.5 times more endothelin-1 than the adipose tissue of lean individuals. Furthermore, this ET-1 generates insulin resistance specifically in visceral, but not in subcutaneous, adipose tissue [119]. This links directly endothelin-1 with insulin resistance and obesity.

5. Animal Models of Metabolic Syndrome: Vascular Function

5.1. The Zucker Obese Rat. The Zucker rat is probably the most commonly used rat model for metabolic syndrome. In 1961, L. M. Zucker and T. F. Zucker discovered that an autosomal recessive mutation in the fatty gene (fa) resulted in obesity [120]. The homozygotes for the mutation (fa/fa) develop obesity because of a defective leptin receptor [121, 122]. Zucker rats develop insulin resistance in addition to obesity, but glycemia remains normal, and they do not develop diabetes [123]. In this aspect, the Zucker rat shares similarities with some of the obese subjects, those who are obese and insulin resistant but are not diabetic. However, the Zucker fatty rat does not mimic the cardiovascular, renal, and neurohumoral changes found in obese humans. For example, this rat has decreased plasma renin activity [124], whereas obese humans often reveal increased renin activity [5]. Also, increased sympathetic activity appears to play a significant role in causing hypertension in obese humans [125], but not in Zucker fatty rats [124]. In addition, conflicting results about whether obese Zucker rats are hypertensive or not compared with their lean controls have been repetitively reported [126]. In a carefully performed study by Hall's group, it was shown that obese Zucker rats suffer no more than 14 mmHg higher than the lean counterparts and that this depends in part on angiotensin II [124].

Regarding vascular responses, much work has been performed in aorta [127–131] and in resistance arteries [132–137] of Zucker rats. Endothelial function assessed in aortic preparations appears to be preserved, or even increased, in young Zucker obese rats compared to the lean rats [128–131]. Andrews et al. use the term *endothelial hyperreactivity* [130] to emphasize the superior endothelial function of Zucker obese rats [131]. For Auguet et al. the increased influence of endothelium in Zucker rats would be related to the absence of atherosclerosis (despite hypercholesterolemia) of these rats. As for resistance arteries, the majority of studies indicate impaired endothelial dysfunction [134–136] and impaired NO-dependent vasodilation [133, 137] in Zucker obese rat arterioles compared to the lean counterpart. By contrast, one study finds equal endothelial function [132].

5.2. The Spontaneously Hypertensive Obese (SHROB) Rat. The obese spontaneously hypertensive rat (SHROB), also known as Koletsky rat, is a rat strain of spontaneous hypertension breeding origin that suffers a nonsense mutation of the leptin receptor gene [138]. This animal was obtained by mating a female SHR of the Wistar-Kyoto strain with a normotensive Sprague-Dawley male. The resulting hybrid offspring was inbred and the obese rat appeared after several generations. The obesity mutation is a recessive trait, designated fa^k, which is a nonsense mutation of the leptin receptor gene resulting in a premature stop codon in the leptin receptor extracellular domain. The SHROB rat carries two fa^k alleles; it is leptin resistant and has circulating leptin levels 30 times higher that the lean counterpart. This mutation makes SHROB rats unable to respond to leptin [139, 140]. This strain arose spontaneously in 1969 in Koletsky's laboratory in Case Western Reserve University School of Medicine (Ohio) [141]. The rat displays obesity, hypertension (although milder than that of their SHR ancestor), hyperinsulinaemia, hyperlipidaemia, and nephropathy, all superimposed on a hypertensive background. Thus, these rats exhibit all the symptoms of metabolic syndrome and are generally regarded as an adequate animal model of this disease [126].

Cardiovascular and renal function has been hardly explored in the SHROB rat. Still, it is known that SHROB rats develop a pronounced diabetic retinopathy. This makes them of special interest for the study of the microvascular complications associated with metabolic syndrome. Huang and coworkers noted that already at 3 months of age they displayed very mild microvascular alterations and did not develop diabetic retinopathy until 10 months of age. Interestingly, control lean SHROB rats also develop diabetic retinopathy [142].

The effect of diet on blood pressure changes has also been studied in these animals. Ernsberger and coworkers observed that drastic fluctuations in the supply of nutrients are not beneficial for blood pressure in these animals. They show that restrictive diet followed by feedback cycles produces blood pressure elevations caused by sympathetic activation and cardiac hypertrophy [143].

Regarding renal and cardiovascular function, it is known that specific binding sites for angiotensin II are decreased in SHROB rats with early glomerular sclerosis, suggesting that angiotensin receptors may be regulated by pathogenic processes in kidneys of these animals [144].

Recently, our group has characterized the macrovascular and microvascular function of this rat strain and the effects of a kind of antidiabetic drugs, glitazones, used in the handling of metabolic syndrome [145]. The SHROB rat clearly suffers

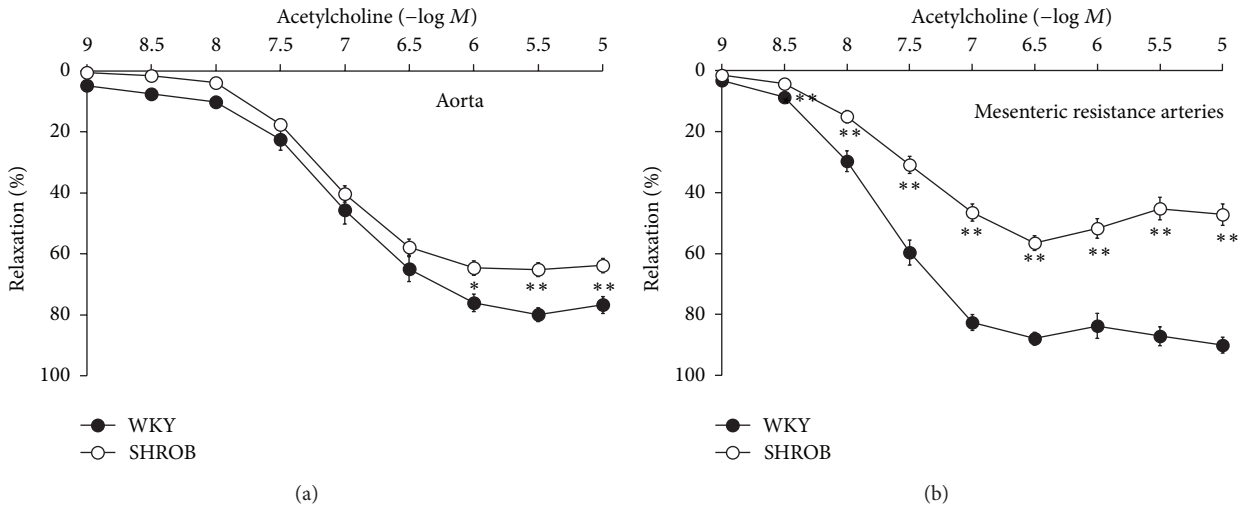

Figure 2: Endothelial function tested by means of acetylcholine responses in aorta (a) and resistance arteries (b) of normotensive (WKY) and metabolic syndrome rats (SHROB). Modified from Mendizábal et al. [145].

macrovascular and most especially microvascular dysfunction (Figures 2(a) and 2(b)). Mesenteric resistance arteries of SHROB rats display a severely impaired endothelium-dependent relaxation due to a failure in the NO system and an abnormally high release of vasoconstrictive prostanoids. These rats also exhibit a dramatic loss in endothelium-independent relaxation, specifically to exogenous NO, suggesting a malfunction of guanylate cyclase. We also showed that drugs used for metabolic syndrome therapy, glitazones, have salutary effects on the endothelial dysfunction of these rats.

5.3. The JCR-LA-cp Corpulent Rat. The JCR-LA-cp corpulent rat is another rat model used to study metabolic syndrome. This rat is homozygous for the autosomal recessive *cp gene (cp/cp)* and is obese, hyperphagic, insulin resistant, hyperinsulinemic, and hypertriglyceridemic [146]. In addition, male JCR-LA-cp rats develop atherosclerosis and myocardial ischemia. Vascular responses and endothelial function were studied by O'Brien and coworkers [146] rendering similar results as for micro- *versus* macrovascular endothelial dysfunction as those of SHROB rats, although the latter displayed a more intense impairment of acetylcholine responses.

5.4. Diet-Induced Obesity. *Stricto sensu*, this model of obesity cannot be always categorized as an animal model of metabolic syndrome because dieting an animal with high fat chow rarely causes the complete cardiovascular and metabolic disease. In some cases, obesity-induced hypertension is achieved [147, 148], but this is not commonplace and most research papers do not report blood pressure values. Other metabolic syndrome symptoms are irregularly reported. For example, hyperinsulinemia or hyperglycemia is found in some studies [149, 150] but not in others [151, 152]. Dyslipemia takes place in some [149, 151] but not all the studies [152]. Hyperleptinemia seems to be common to all [150–152].

However, keeping in mind the enormous epidemiological dimension of overweight, obesity, and obesity-associated cardiovascular problems (i.e., cardiometabolic syndrome), much research and effort have been performed in these kind of rat or mouse models regardless of whether the animal develops or not a complete metabolic syndrome. Another factor in favor of diet-induced obesity animal models is that they are more human-like models, where the obesity is based on an excess intake of calories, whilst genetic models deficient in the leptin receptor or leptin synthesis are not representative of the human pathophysiology of obesity. Obesity in rodents can also be induced with the so-called *cafeteria diet*. In this model, animals have a choice of various energy-dense foods. The advantage to this approach is that the diet is palatable and the propensity to overeat is larger than that for the high-fat chow diet. Needless to say is that this is the most similar to the human dietary situation [153].

Regarding vascular function, the vast majority of studies have reported alterations. Endothelial function, assessed by acetylcholine responses, has been found altered in most cases. For example, in a cafeteria diet model reported by Naderali et al., a negative association between plasma lipid levels and reduction in acetylcholine-induced vasorelaxation was found [151]. Furthermore, a study in obese people showed that weight loss improves endothelial function together with various metabolic syndrome symptoms [154]. Hypercontractility, albeit less studied, has been reported in rats made hypertensive through the diet [147, 148]. In recent times, a large amount of studies have been focused on the effects that the local adiposity surrounding blood vessels (the so called PVAT) has on smooth muscle cell contractility and endothelial function [77]. Adipose tissue specifically located close to blood vessels exhibits a proinflammatory phenotype compared to other depots such as the subcutaneous one [155]. This phenotype is aggravated after a high fat feeding suggesting that PVAT is very sensitive to the effects of excess dietary fat [155]. In obese rats, including diet-induced

obesity rats, it has been repetitively shown that PVAT causes endothelial dysfunction via proinflammatory cytokines such as TNFα [156] or monocyte chemotactic protein-1 [157] as well as through oxidative stress [148, 157]. Actually, Dobrian et al. report on a rat model of obesity-induced hypertension that this increase in vascular oxidative stress is associated with an increase in vascular NO production and NO synthase activity [148]. Furthermore, Jebelovszki et al. demonstrated that diet-induced obesity increases vascular smooth muscle sensitivity to NO through an activation of guanylate cyclase [149]. To have a whole picture of the biology of NO in obesity it is important to consider also that adipocytes can express NO synthase and that this expression is upregulated in obesity [158]. The adipocytic upregulation of NO synthase contrasts with the endothelial downregulation of this enzyme described by Ma et al. in diet-induced obesity rats in which this downregulation finely correlates with the vascular dysfunction they find in their own experiments [159] and, in general, in those of others [152–157]. This apparent contradiction can be explained as follows. In nonobese individuals, PVAT would have a vascular protective and beneficial role [152]. During the onset of obesity, several adaptive mechanisms within the vessel wall [149] and within PVAT itself [152] are activated. Regarding the latter, Gil-Ortega et al. have published interesting data showing the existence of an adaptive NO overproduction by PVAT during early diet-induced obesity and propose that, at some time point during obesity development, PVAT switches from a vascular protective influence to a deleterious one [152].

6. Future Directions

The pathophysiology of metabolic syndrome has become very complex. We have reviewed some of the pathophysiological aspects that affect vascular function: insulin, sympathetic system, endothelium, perivascular fat, and adipokines. The animal models in use have important limitations that need to be compensated with clinical studies. Translational research, in which animal studies are designed and carried out together with clinical investigation, is of special value. It is also highly important to merit basic science studies designed to unravel specific pathways, messengers, and intermediates of metabolic syndrome. While the era of endothelium and endothelium-derived substances has passed its summit, the age of perivascular adipocytes and adipokines is coming with a strong impulse.

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Research Article

Mapping Metabolic Brain Activity in Three Models of Hepatic Encephalopathy

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Cirrhosis is a common disease in Western countries. Liver failure, hyperammonemia, and portal hypertension are the main factors that contribute to human cirrhosis that frequently leads to a neuropsychiatric disorder known as hepatic encephalopathy (HE). In this study, we examined the differential contribution of these leading factors to the oxidative metabolism of diverse brain limbic system regions frequently involved in memory process by histochemical labelling of cytochrome oxidase (COx). We have analyzed cortical structures such as the infralimbic and prelimbic cotices, subcortical structures such as hippocampus and ventral striatum, at thalamic level like the anterodorsal, anteroventral, and mediodorsal thalamus, and, finally, the hypothalamus, where the mammillary nuclei (medial and lateral) were measured. The severest alteration is found in the model that mimics intoxication by ammonia, followed by the thioacetamide-treated group and the portal hypertension group. No changes were found at the mammillary bodies for any of the experimental groups.

1. Introduction

Portal hypertension is one of the main complications of human cirrhosis that frequently leads to a neuropsychiatric disorder known as hepatic encephalopathy (HE). The genesis of portal hypertension implies an increase in vascular resistance that can occur at any level within the portal venous system [1]. Elevated blood flow and brain ammonia levels have been strongly implicated in the pathogenesis of HE [2]. Ammonia is a common etiological factor in HE as well as in various hyperammonemic conditions, including inborn errors of the urea cycle, Reye's syndrome, valproate toxicity, idiopathic hyperammonemia, and other conditions [3–6]. Elevated ammonia and its chief metabolite, glutamine, are believed to be important factors responsible for altered cerebral functions, including multiple neurotransmitter system failures, altered bioenergetics, and oxidative stress [7].

Likewise, patients with liver disease have HE, which incorporates a spectrum of manifestations including psychomotor dysfunction, increased reaction time, sensory abnormalities, and poor concentration [8]. Depending on the definition used, HE prevalence varies between 30–84% in patients with cirrhosis [9], a common disease in Western countries [10]. In humans, few studies have been carried out on memory alterations in patients with cirrhosis who develop HE, and, although some authors argue that memory disturbances are not a major symptom of HE [11], others state that patients with HE present clear mnesic alterations. Hence, Bahceci et al. [12] found a poorer performance in several memory tests in patients with cirrhosis, whereas Ortiz et al. [13] showed a learning deficit and impairment in long-term memory.

Taken together, liver disease, portal hypertension, and hyperammonemia appear to be the main contributing factors that lead to the occurrence of HE.

According to the proposal of Butterworth et al. [14], HE is reclassified into different types, depending on its origin or cause. Type B concerns HE related to portosystemic shunt,

which does not necessarily involve any hepatocytic alteration and includes portocaval shunt, partial ligature of the portal vein, and triple portal vein ligation [15]. Type C is associated with chronic liver failure (cirrhosis). As HE can be derived from different causes, diverse experimental models have been developed to reproduce the characteristics of HE as closely as possible and to study brain dysfunction in this syndrome [14]. In this study, we try to isolate the main factors leading to HE through the use of three experimental models: portal hypertension, hyperammonemia, and thioacetamide. Portal hypertension by triple portal vein ligation [16] involves collateral circulation but with a discrete hepatocellular insufficiency. The model of hyperammonemia [17] allowed us to study the effect of ammonia as a toxic brain substance but it lacks also liver failure [18]. Therefore, to explore cirrhosis, a model of chronic thioacetamide administration was used [19].

Difficulties learning diverse types of tasks have been shown in these experimental models, such as classical conditioning [20], conditional discrimination [21, 22], reference [23], and working memory [24]. However, less is known about the specific role of liver failure, portal hypertension, and hyperammonemia in the development of bioenergetic brain disturbances.

The aim of this study is to elucidate the differential contribution of these leading factors to the oxidative metabolism of diverse brain limbic system regions frequently involved in memory process by histochemical labelling of cytochrome oxidase (COx). COx is a mitochondrial enzyme involved in the phosphorylation process that generates ATP. This energy is used to maintain the resting membrane potential and the synthesis of molecules and neurotransmitters, among other functions [25]. Because metabolic activity is tightly coupled with neuronal activity, this technique can be used as an index of regional functional activity in the brain, reflecting changes in tissue metabolic capacity induced by sustained energy requirements of the nervous system associated with learning [23, 26].

2. Material and Methods

2.1. Subject. A total of 28 male adult Wistar rats were used (230–260 g at the start of the experiments) from the animalarium of Oviedo University. All the animals had ad libitum access to food and tap water and were maintained under standard laboratory conditions (20–22°C, 65–70% relative humidity, and a 12 h light/dark cycle). The procedures and manipulation of the animals used in this study were carried out according to the European Parliament and the Council of the European Union 2010/63/UE and were approved by the Oviedo University committee for animal studies.

2.2. Procurement of Experimental Models. The animals were randomly distributed into 3 groups: portal hypertension (PH group, $n = 12$), hyperammonemia (HA group, $n = 8$), and animals with cirrhosis by administration of thioacetamide (TAA group, $n = 8$). The surgical procedures and protocols used for the different experimental models are described below.

2.2.1. Portal Hypertension. The portal hypertension model was carried out under induction of anaesthesia by i.m. injection of ketamine (100 mg/kg) and xylacine (12 mg/kg). A midline abdominal incision was performed and a part of the intestinal loops was gently shifted to the left and covered with saline-moistened gauze. The portal vein was isolated along its length. Portal hypertension was produced by triple partial ligation [16]. Three stenosing ligatures were performed in the superior, medial, and inferior portions of the portal vein, respectively, and maintained in position by the previous fixation of the ligatures to a sylastic guide. The stenoses were also calibrated by a simultaneous ligature (3–0 silk) around the portal vein and a 20 G needle. The abdominal incision was closed in two layers with an absorbable suture (polyglycolic acid) and 3–0 silk. With respect to postsurgical care, the rats were maintained close to a source of heat until they recovered consciousness (10–15 min) to avoid postoperative hypothermia. Brains were assessed 45 days later.

2.2.2. Thioacetamide. The method used to produce cirrhosis was weekly administration of thioacetamide (Sigma, Germany) in drinking water as described by Li et al. [19]. The thioacetamide (TAA) was administered for 12 weeks and its concentration was modified weekly depending on the animals' weight gain or weight loss. The initial concentration of TAA was 0.03%. After twelve weeks of treatment, they were placed in groups of 5 rats per cage and they were assessed after two weeks. During this period, the animals received TAA at 0.04%.

2.2.3. Hyperammonemia. The method used to produce hyperammonemia was performed according to Azorín et al. [17]. Briefly, the animals were fed with a standard diet supplemented with ammonium acetate ad libitum for up to 27 days.

2.3. Cytochrome Oxidase Histochemistry. 90 minutes after the performance of a spatial reference memory task, animals were decapitated, and the brains were removed intact, frozen rapidly in isopentane (Sigma-Aldrich), and stored at −40°C. Coronal sections (30 μm) of the brain were cut at −20°C in a cryostat (Leica CM1900, Germany) and were mounted on slides.

We used a quantitative COx histochemistry described by Gonzalez-Lima and Cada [25]. To quantify enzymatic activity and to control staining variability across different baths of staining, sets of tissue homogenate standards obtained from Wistar rat brains were included with each bath of slides. These standards were cut at different thicknesses (10, 30, 40, and 60 μm).

Sections and standards were incubated for 5 min in 0.1 M phosphate buffer with 10% w/v sucrose and 0.5% v/v glutaraldehyde, pH 7.6. After this, four baths of 0.1 M phosphate buffer with 10% w/v sucrose were given for 5 min each. Then 0.05 M Tris buffer, pH 7.6, with 275 mg/L cobalt

chloride, 10% w/v sucrose, and 0.5% v/v dimethylsulfoxide were applied for 10 min, to enhance staining contrast. Subsequently, sections and standards were incubated in a solution of 0.06 g cytochrome c (Sigma, St. Louis, MO, USA), 0.016 g catalase, 40 g sucrose, 2 mL dimethylsulfoxide, and 0.4 g diaminobenzidine tetrahydrochloride in 800 mL of 0.1 M phosphate buffer, at 37°C for 1 h. The reaction was stopped by fixing the tissue in buffered formalin for 30 min at room temperature with 10% w/v sucrose and 4% v/v formalin. Finally, the slides were dehydrated in series of ethanol baths (from 30% to 100% v/v ethanol), cleared with xylene, and coverslipped with Entellan (Merck, Germany).

2.3.1. Quantification. Quantification of COx histochemical staining intensity was done by densitometric analysis using a computer-controlled image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England) made up of a high-precision illuminator, a digital camera, and a computer with specific software for image analysis. The mean optical density (OD) of each structure was measured on the right side of the bilateral structures using three consecutive sections of each animal. In each section, four nonoverlapping readings were taken using a square-shaped sampling window that was adjusted for each region size. A total of twelve measurements were taken per region. These twelve measurements were averaged to obtain one mean per region for each subject. OD values were then converted to COx activity units, determined by the enzymatic activity of the standards which were measured spectrophotometrically [25]. Measurements were performed by an investigator blind to the groups.

The regions of interest were anatomically defined according to Paxinos and Watson (2005) [27]. The regions of interest and the distance in mm of the regions counted from bregma were: +3.20 mm for prefrontal cortex (the infralimbic cortex and prelimbic cortex (IL and PL, resp.), +2.28 mm for ventral striatum (accumbens core and shell, AcC and AcS), −1.40 for the anterodorsal thalamus (ADT), the anteroventral thalamus (AVT), and the mediodorsal (MDT), −1.20 mm for the CA1, CA3, and the dentate gyrus (DG) subfields of the dorsal hippocampus, and +4.52 mm for the medial part of the medial mammillary nucleus (mMM), the lateral part of the medial mammillary nucleus (lMM), and the lateral mammillary nucleus (LM). See Figure 1.

2.4. Data Analysis. All data were analyzed in the Sigma-Stat 3.2 program (Systat, Richmond, USA) and were expressed as mean ± SEM. A one-way analysis of variance was performed (factor: group). When a significant effect was found post hoc paired Tukey's test was carried out. The results were considered as statistically significant if $P < 0.05$.

3. Results

The post hoc paired Tukey's tests showed differences between groups in their prefrontal cortex COx activity. The HA group showed higher activity than the PH and TAA groups in the PL ($F2, 24 = 47.674$) ($P < 0.001$), and the TAA group also showed higher activity than the PH group ($P < 0.001$). In the IL, the same difference between the HA group and the other groups was found ($F2, 24 = 53.649$, $P < 0.001$). In the ventral striatum, the same pattern of metabolic activity was found in the AcC and AcS, with the HA group showing higher activity, followed by the TAA group, which differed from the PH group ($F2, 25 = 69.662$, $P < 0.001$; $F2, 25 = 76.819$, $P < 0.01$, resp.). These differences were also found in the ADT ($F2, 25 = 63.441$, $P < 0.001$). However, in the AVT ($F2, 25 = 13.610$, $P < 0.05$) and MDT ($F2, 25 = 27.970$, $P < 0.001$), differences were only found when comparing the HA group with the other experimental groups, with the former group being the most highly activated. Examining the dorsal hippocampus, in the DG, the HA group showed higher activity than the PH and TAA groups ($F2, 25 = 47.402$, $P < 0.01$), and the TAA group showed greater activity than the PH group ($P < 0.001$). Similarly, in CA3, the HA group was the most highly activated in relation to the other two groups ($F2, 25 = 63.059$, $P < 0.001$), and the TAA group showed lower activity than the PH group ($P < 0.001$). In CA1, the highest metabolic activity was found in the HA group, which presented differences with the PH and the TAA groups ($F2, 25 = 16.954$, $P < 0.001$). Finally, no differences were found in any of the mammillary nuclei explored: mMM ($F2, 24 = 0.109$, $P = 0.898$), lMM ($F2, 24 = 1.368$, $P = 0.274$), and LM ($F2, 23 = 0.178$, $P = 0.838$). See Table 1.

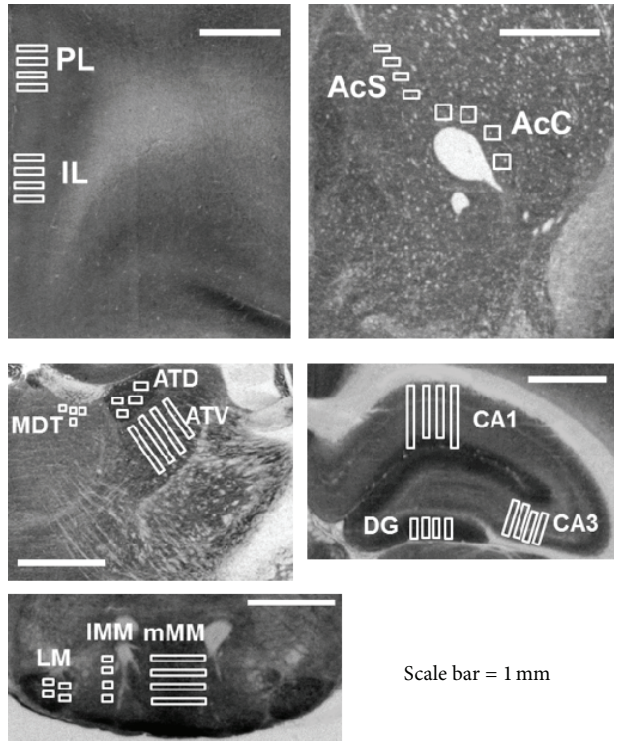

Figure 1: Cytochrome oxidase (COx) histochemistry in the sampled regions in which the squares used to take the measures were presented. Prelimbic (PL) and infralimbic (IL) cortex, accumbens core and shell (AcC and AcS), thalamic nuclei (ADT, AVT, and MDT), dorsal hippocampus (CA1, CA3, and DG) and Mammillary nuclei (mMM, lMM, and LM). Scale bar: 1 mm.

Table 1: Metabolic activity of the selected brain regions in the studied groups.

	PH	HA	TAA
Prefrontal cortex			
PL	18.364 ± 0.460[a,b]	26.781 ± 1.032	22.591 ± 0.379[a]
IL	18.470 ± 0.504[a]	26.988 ± 0.916	19.432 ± 0.401[a]
Ventral Striatum			
AcC	21.362 ± 0.395[a,b]	31.812 ± 1.032	25.552 ± 0.498[a]
AcS	22.210 ± 0.748[a,b]	36.940 ± 1.227	32.409 ± 0.715[a]
Thalamic nuclei			
ADT	30.525 ± 0.736[a,b]	50.930 ± 2.024	40.025 ± 1.302[a]
AVT	24.717 ± 0.525[a]	31.753 ± 1.412	27.631 ± 1.120[a]
MDT	19.307 ± 0.879[a]	27.361 ± 0.809	18.242 ± 0.921[a]
Hippocampus			
DG	23.413 ± 0.500[a,b]	39.550 ± 1.75	32.663 ± 1.739[a]
CA3	16.318 ± 0.488[a,b]	22.633 ± 0.763	12.592 ± 0.568[a]
CA1	18.128 ± 0.707[a]	24.820 ± 0.915	18.250 ± 0.915[a]
Mammillary nuclei			
mMM	22.949 ± 1.151	23.490 ± 1.336	23.769 ± 1.479
lMM	17.643 ± 0.950	16.792 ± 0.633	15.777 ± 0.597
LM	25.923 ± 1.113	26.624 ± 1.246	26.665 ± 0.489

Values are mean ± SEM.
PH: portal hypertension, HA: hyperammonemia, TAA: thioacetamide.
[a] $P < 0.05$ statistically significant value in relation to HA.
[b] $P < 0.05$ statistically significant value in relation to TAA.

4. Discussion

The rationale for the present study arose from evidence that cerebral metabolic rate for glucose has consistently been reported to be decreased in patients with chronic HE, suggesting that hypometabolism contributes to the neuropsychiatric symptoms commonly observed in HE. In fact, it has been shown that cerebral oxygen metabolism and blood flow were decreased in cirrhotic patients with HE [28], and cognitive deficits are closely related to aberrant baseline brain activity measured by fMRI in these patients [29]. However, the existence of a differential contribution of factors, such as portal hypertension, hyperammonemia, and liver disease, to brain metabolic activity has not yet been explored. In this study, we demonstrated that each of these factors affects brain energy requirements to a different extent.

Several studies have assessed these experimental models in a spatial reference memory task. Whereas the portal hypertension model showed only mild impairment in this learning process [23], hyperammonemia and liver failure models were severely affected (Arias et al., unpublished results [30]).

Traditionally, the hippocampus has been widely shown to be involved in reference memory [31]. Specifically, it is important in establishing allocentric relations [32] and also seems to be responsible for correct goal recognition [33]. Together with this brain structure, prefrontal cortex, ventral striatum, anterior thalamus, and mammillary bodies cooperate in diverse memory processes and learning tasks [34, 35]. The role of the prefrontal cortex in the organization of spatially directed behaviours has been shown both in primates and humans. In rats, the main evidence comes from lesion studies in performance of various spatial tasks such as spatial alternation, spatial reversal, elimination in the radial maze, and navigation in the Morris water maze [36]. The connections between this prefrontal cortex and ventral striatum play a role in the transformation of route planning into motor response in memory tasks [37]. Likewise, the prefrontal cortex receives projections from the anterior thalamic nucleus [38], damage to which impairs allocentric memory tasks that are also disrupted by hippocampal dysfunctions [39]. The mammillary nuclei, which can be subdivided into the medial and lateral parts, are connected with the hippocampus and send projections to the anterior thalamus [40] and are involved in spatial memory processes [41, 42].

When we wondered about the possible time-dependent involvement of these structures in the spatial reference memory, we found that most of them were differentially implicated at the beginning and the conclusion of the task in nonpathological conditions [43]. That is, some structures, such as the anterior thalamic nuclei and the mammillary bodies, seem to be more involved in early task acquisition, but it is necessary for these structures to reduce their energy requirements in order to successfully complete the task. But other structures, such as DG, CA3, and the prefrontal cortex, showed an opposite pattern [43].

In view of this tendency, we were willing to explore the metabolic activity of the main contributing factors of HE. Accordingly to our expectations, the portal hypertension group reduced its metabolic activity in almost all studied

regions compared to the other groups. A possible explanation for this behaviour, so similar to that of nonpathological subjects, is that, in the PH model, portosystemic collaterals develop as a consequence of high pressure in the portal vein and ameliorate the increased resistance [1, 44]. However, a different situation takes place in the HA group, in which the energetic demands increase above conventional levels. This increase in oxygen utilization could be due to an inflammation in these areas of the central nervous system [45]. Inflammation will increase the use of oxygen, represented by excess production of reactive oxygen and nitrogen species, which cause oxidative and nitrosative stress [46–48].

Several studies have shown that once ammonia crosses the blood brain barrier, it is immediately used to amidate glutamate to glutamine in the astrocytes in order to prevent its toxicity from damaging the neurons. But this depletion of the substrate of the tricarboxylic acid cycle together with the inhibition by the ammonia to rate limit enzymes such as pyruvate dehydrogenase and α-ketoglutarate will slow down overall oxidative metabolism, leading to depletion of energy-rich phosphate compounds [49]. Moreover, glutamatergic neurotransmission suffers disturbances owing to the fact that ammonia impairs the induction of NMDA receptors, which alters the neural glutamate-nitric oxide cyclic GMP pathway, in turn, involved in learning and memory [20, 21].

At the same time, glutamine, which is an osmotically powerful substance, increases due to ammonia in the brain and can provoke a rapid brain edema, triggering intracranial hypertension. During liver failure, high arterial ammonia levels lead to accumulation in the brain [50] and exert numerous deleterious effects, contributing to the clinical presentation of HE. Subsequent studies carried out in various animal models of acute and chronic HE, such as our TAA model, have reported altered glucose utilization similar to the HA models [51]. But, whereas patients with liver cirrhosis have a compensatory mechanism that prevents the occurrence of brain edema and intracranial hypertension [18], the hyperammonemic brain cannot establish these mechanisms.

Another possible mechanism for impaired energy metabolism in HA is the mitochondrial permeability transition of the inner membrane, which has a critical linkage with disturbed cerebral energy metabolism and oxidative/nitrosative stress [51]. Similar to free radicals, nitric oxide increases in the brain of the TAA-administration rat model [52]. A possible explanation for the alteration of COx activity would be the increased level of oxidative stress [45]. Liver disease is associated with the production of free radicals that override the balance between oxidative stress and antioxidative mechanisms, leading to oxidative stress. Oxidative stress could play a role in the pathogenesis of HE, due to the fact that the brain of patients with HE is particularly susceptible to oxidative damage [47]. Peroxidase damage and increased oxidative stress have been reported in neural membranes of rats with thioacetamide-induced HE [53] and in diverse central nervous system regions of the same animal model [54]. Brain oxidative and nitrosative (superoxide anion with nitric oxide produces peroxynitrite) stress in cirrhotic rats could cause neural mitochondrial damage and defective oxidative phosphorylation, which is reflected in impaired COx activity. Therefore, oxidative and nitrosative stress caused activation of the nuclear factor KB, which is the major inducer of proinflammatory cytokines and chemokines.

The differences found in bioenergetic requirements could indicate a differential affectation of the neural networks underlying different behaviours, so a broad field of future research opens up.

In conclusion, the results obtained show that there is a differential contribution of portal hypertension, hyperammonemia, and liver disease to the brain metabolic dysfunction associated with HE. The most interesting finding is that the alterations in metabolic brain activity do not develop equally in the three models. The severest alteration is found in the model that mimics intoxication by ammonia, where the main cerebral structures have to make a big effort in comparison with the other two experimental models.

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Research Article

Nox2 Deficiency Prevents Hypertension-Induced Vascular Dysfunction and Hypertrophy in Cerebral Arterioles

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Oxidative stress is involved in many hypertension-related vascular diseases in the brain, including stroke and dementia. Thus, we examined the role of genetic deficiency of NADPH oxidase subunit Nox2 in the function and structure of cerebral arterioles during hypertension. Arterial pressure was increased in right-sided cerebral arterioles with transverse aortic banding for 4 weeks in 8-week-old wild-type (WT) and Nox2-deficient (-/y) mice. Mice were given N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg) or vehicle to drink. We measured the reactivity in cerebral arterioles through open cranial window in anesthetized mice and wall cross-sectional area and superoxide levels *ex vivo*. Aortic constriction increased systolic and pulse pressures in right-sided carotid arteries in all groups of mice. Ethidium fluorescence showed increased superoxide in right-sided cerebral arterioles in WT, but not in Nox2-/y mice. Dilation to acetylcholine, but not sodium nitroprusside, was reduced, and cross-sectional areas were increased in the right-sided arterioles in WT, but were unchanged in Nox2-/y mice. L-NAME reduced dilation to acetylcholine but did not result in hypertrophy in right-sided arterioles of Nox2-/y mice. In conclusion, hypertension-induced superoxide production derived from Nox2-containing NADPH oxidase promotes hypertrophy and causes endothelial dysfunction in cerebral arterioles, possibly involving interaction with nitric oxide.

1. Introduction

Chronic hypertension is a major risk factor of vascular disorders. Profound vascular functional and structural changes occur in many disease states, and emerging evidence suggests that oxidative stress has a major role in mediating these changes [1]. For example, increased oxidative stress has been shown in hypertension-induced vascular diseases, stroke and subcortical vascular dementia [2–4]. Moreover, oxidative stress from various sources has been implicated in endothelial dysfunction and structural remodeling in the cerebral vasculature [5, 6].

Although there are many sources of reactive oxygen species (ROS), the primary source of superoxide production in the vascular wall is thought to be NADPH oxidase [7]. NADPH oxidase consists of a membrane-bound *b*-type cytochrome composed of 91 and 22 kDa subunits (referred to as $gp91^{phox}$ (also known as Nox2) and $p22^{phox}$, resp.), and three cytosolic proteins ($p47^{phox}$, $p67^{phox}$, and $p21^{rac}$) [7]. While functional forms of NADPH oxidase have been demonstrated throughout the vasculature, there are subtle, but important, structural differences with respect to its subunits depending on vessel size and region. For example, it appears that Nox2 may play a role in NADPH oxidase activity in vascular muscle of small resistance arteries, whereas homologues of Nox2, such as Nox1 and Nox4, may be more important in large cerebral arteries [8, 9]. Furthermore, a number of studies showed that angiotensin II-induced impairment of endothelial function and reduced cerebral blood flow are restored in Nox2-deficient (-/y) mice [10, 11]. These findings highlight the importance of Nox2-containing NADPH oxidase in the pathology of hypertension in the cerebral circulation.

Cerebral resistance arteries and arterioles play critical roles in controlling local cerebral blood flow [12]. It is, therefore, important to understand the mechanism for functional

and structural changes during chronic hypertension in these small brain vessels. In an effort to determine the source of ROS in cerebral arterioles during hypertension, the first goal of this study was to examine the hypothesis that Nox2-derived ROS results in vascular dysfunction and hypertrophy in cerebral arterioles during hypertension. We used a transverse aortic banding procedure to increase cerebral vascular pressure and oxidative stress in the right side of the brain [13, 14]. An advantage of this model is that the left cerebral hemisphere remains normotensive relative to the right side, and thus vessels in the left hemisphere can be used as normotensive controls.

Nitric oxide (NO) is a major mediator of endothelium-dependent dilation and inhibits mitogenesis and proliferation of vascular smooth muscle cells [15]. NO readily reacts with superoxide; thus, the local concentration of superoxide is an important determinant of the biological availability of NO [16]. A previous study has demonstrated the relevance of the NO-dependent pathway in endothelial dysfunction and hypertrophy in cerebral vasculature [17]. Thus, our second goal was to examine the hypothesis that the NO-dependent pathway plays a role in Nox2-derived ROS-induced dysfunction and hypertrophy of cerebral arterioles during chronic hypertension.

2. Methods

2.1. Animals. Nox2-/y and wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in pathogen-free facility at 24°C, exposed to 12 hours of light (lights on at 06:00, off at 18:00) and allowed free access of food and fluid. All animals were studied at 13 to 15 weeks of age. Procedures followed in this study were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

2.2. Transverse Aortic Banding. Increased pressure in the proximal aorta in all animals was induced by means of thoracic aortic banding using the method described previously [18]. Briefly, mice were anesthetized with ketamine (100 mg/kg, i.p.) plus xylazine (5 mg/kg, i.p.), intubated with 20-gauge tubing and ventilated (Harvard Apparatus Rodent Ventilator, model 687) at 100 breaths per minute (0.1 mL tidal volume). Thoracotomy was created at the second intercostal space. The transverse aortic arch was ligated (7–0 Prolene) between the innominate and left common carotid arteries with an overlying 27-gauge needle, and then the needle was removed, leaving a discrete region of stenosis. The 24 h and 1-week survival rate were each higher than 90%.

2.3. L-NAME Treatments. L-NAME (10 mg/kg/day, 4 weeks) was given in drinking water to WT ($n = 16$) and Nox2-/y ($n = 16$) mice. This dose regimen has been shown to induce hypertrophy in cerebral arterioles [17]. The treatment was started the same day after aortic banding. We replaced freshly prepared L-NAME solution everyday or every other day. We adjusted the concentration of L-NAME every time based on the volume an individual mouse drank.

2.4. Determination of Blood Pressures in Conscious Animals. Systemic arterial blood pressures were measured in 6 mice from each group using an automated tail-cuff device (Visitech Systems BP-2000, Apex, NC, USA). Mice were placed in specifically designed mouse holders that allow measurement of systolic blood pressure under resting conditions. Mice were trained for 5 days, and then blood pressure was measured at days 0 (baseline), 7, 14, 21, and 28 of treatment. Each day, 30 measurements were made and averaged for each mouse.

2.5. Determination of Cerebral Arteriolar Diameter and Structure. Four weeks after aortic banding, we measured diameter in first-order arterioles on the surfaces of the right and left cerebral hemispheres through an open skull preparation as described in detail previously ($n = 8$ in each group) [19, 20]. Cerebral arterioles were monitored through a microscope connected to a closed-circuit video system with a final magnification of ×356. Arteriolar diameter was measured from digitized images of arterioles using NIH Image version 1.62 (National Institute of Health, USA). About 30 minutes after completion of craniotomy, cerebral arterioles were exposed to acetylcholine (ACh, 10^{-5} M) dissolved in artificial cerebral spinal fluid (CSF) for 5 min. Arteriolar diameters were measured and drug was washed by CSF for 5 min. The procedure was repeated with sodium nitroprusside (SNP, 10^{-7} M). In addition, systemic arterial pressure was measured continuously via catheters inserted into the right and left common carotid arteries.

To determine whether increases in arterial pressure that result from transverse aortic banding are limited to the right side of the brain, pressure was measured in right- and left-sided first-order cerebral arterioles in a separate group of anesthetized WT mice ($n = 6$) using a servo-null system as described in detail previously [19, 20]. The mice had undergone the transverse aortic banding procedure 4 weeks before measuring arteriolar pressure. Systolic (SP), diastolic (DP), mean (MP), and pulse (PP) pressures were significantly higher ($P < 0.05$) in right-sided (62 ± 6, 35 ± 2, 44 ± 3, and 28 ± 4 mmHg; SP, DP, MP, and PP, resp.) than in left-sided cerebral arterioles (39 ± 3, 28 ± 2, 32 ± 2, and 10 ± 1 mmHg; SP, DP, MP, and PP, resp.). Furthermore, the levels of SP, DP, MP, and PP in left-sided arterioles in aortic banded mice were similar to those we observed previously in normotensive WT mice [5, 6].

In another set of animals ($n = 8$ in each group), structural characteristics were studied using the same open cranial window technique. After the baseline diameters were measured, arterioles then were suffused with CSF-containing EDTA (67 mM), which produces maximal dilation of cerebral arterioles [21]. Arterioles were fixed at physiological pressure *in vivo* by suffusion of vessels with glutaraldehyde fixative (2.25% glutaraldehyde in 0.1 M cacodylate buffer) while maintaining cerebral arteriolar pressure at baseline levels. After the anesthetized animal was euthanized using overdose sodium pentobarbital, cerebral arteriolar segments and carotid arteries were removed, processed, and embedded in Spurr's low-viscosity resin while maintaining cross-sectional orientation. Cross-sectional area (CSA) of the vessel wall

was determined histologically using a method described previously [21].

2.6. Determination of Superoxide in Cerebral Arterioles. In another set of animals ($n = 8$ in each group), superoxide levels were evaluated *in vitro* in 6–8 μm thick frozen sections of unfixed right- and left-sided cerebral arterioles using hydroethidine-based (2 μM hydroethidine) confocal microscopy as described previously [22]. Laser settings were identical for the acquisition of all images, and vessels from WT and Nox2-/y mice were processed and imaged in parallel. Relative increases in ethidium fluorescence were determined and normalized to the cross-sectional area of the vessel wall.

2.7. Drugs. ACh, SNP, and L-NAME were purchased from Sigma (St. Louis, MO, USA). ACh and SNP were dissolved in artificial CSF. L-NAME was dissolved in distilled water.

2.8. Statistical Analysis. Analysis of variance was used to compare blood pressure, cerebral arteriolar diameters, cross-sectional areas, and superoxide levels of the vessel wall. Probability values were calculated using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). Values were presented in mean ± SEM and were considered different when $P < 0.05$ using post hoc Bonferroni test.

3. Results

3.1. Nox2 Deficiency Inhibits Hypertension-Induced Superoxide Production in Cerebral Arteriole. To determine if Nox2-containing NADPH oxidase is responsible for hypertension-induced superoxide production, levels of superoxide were determined in cerebral arterioles from WT and Nox2-/y mice by ethidium fluorescence. Representative micrographs show that fluorescence of ethidium was higher in right- than left-sided cerebral arterioles in WT mice (Figure 1(a), left panel). In contrast, fluorescence in right-sided arterioles did not appear to be increased in Nox2-deficient mice (Figure 1(a), right panel), and fluorescence in left-sided arterioles appeared to be lower in Nox2-deficient mice than in WT mice. Semiquantification of ethidium signal confirmed that levels of fluorescence were higher in right- than left-sided cerebral arterioles in WT mice (Figure 1(b)) and similar in right-and left-sided arterioles in Nox2-/y mice (Figure 1(c)). These findings suggest that Nox2 is the major source of hypertension-induced superoxide in cerebral arterioles.

3.2. Nox2 Deficiency Did Not Alter Blood Pressure. SDs measured under conscious conditions by a tail-cuff method prior to L-NAME treatment were similar in WT and Nox2-/y mice (Figure 2). Aortic banding did not alter conscious SP in any of the animals. Blood pressure of WT and Nox2-/y mice was increased in the last two weeks of L-NAME treatment. Response to L-NAME was similar in both strains.

Pressures were measured in right and left carotid arteries in anesthetized mice to confirm that transverse aortic banding was successful. SP and PP, but not DP and MP, were significantly increased by similar levels in right- compared to left-sided carotids in untreated WT and untreated Nox2-/y mice (Table 1).

3.3. Nox2 Deficiency Prevents Hypertension-Induced Endothelial Dysfunction in Cerebral Arterioles. To test whether endothelial dysfunction induced by hypertension is Nox2-dependent, dilator responses to ACh and SNP were studied. Dilator response to ACh was significantly decreased in right-sided cerebral arterioles relative to left-sided arterioles in untreated WT mice, suggesting an endothelial dysfunction on the hypertensive side (Figure 3(a)). Response to SNP was similar in both sides of WT mice, indicating that aortic banding did not affect smooth muscle contractility (Figure 3(b)). In untreated Nox2-/y mice, responses to Ach were restored in right-sided cerebral arterioles comparable to that of the left side, suggesting normal endothelial function. Treatment with L-NAME reduced dilator responses in left-sided cerebral arterioles to ACh, but not SNP, in both WT and Nox2-/y mice. Moreover, L-NAME blunted dilator responses to ACh in right-sided arterioles in Nox2-/y mice.

3.4. Nox2 Deficiency Prevents Hypertension-Induced Hypertrophy in Cerebral Arterioles. To determine whether Nox2 contributes to hypertension-induced hypertrophy in cerebral arterioles, we measured CSA of the arteriolar wall. CSA of the arteriolar wall was greater in right-, than in left-sided, cerebral arterioles in untreated WT mice, but not in untreated Nox2-/y mice (Figure 4(a)). Treatment with L-NAME increased CSA in left-sided, but not right-sided cerebral arterioles, in WT mice. In contrast, L-NAME did not produce hypertrophy in either right- or left-sided arterioles in Nox2-/y mice, which suggests an important role for Nox2-dependent production of ROS in the development of hypertension-induced cerebral arteriolar hypertrophy. In contrast to cerebral arterioles, Nox2 deficiency did not prevent increases in CSA of the vessel wall in carotid arteries (Figure 4(b)), suggesting that Nox2 does not contribute to hypertension-induced hypertrophy in larger conduit arteries.

4. Discussion

Chronic hypertension has profound impacts on the vasculature and is a known risk factor for stroke and dementia. It is important to understand the mechanism of vascular changes during chronic hypertension, particularly in smaller resistance arterioles because they provide substantial vascular resistance and are important in controlling local blood flow [12]. In this study, we used a transverse aortic banding model to increase blood pressure to the right, but not the left, side of the brain to study mechanisms of vascular dysfunction and structural remodeling in chronic hypertension. There are several important findings. First, superoxide levels were increased in the hypertensive side of the brain in WT, but not in Nox2-/y mice. This result suggests that Nox2-containing NADPH oxidase is the major source of superoxide in cerebral arterioles during hypertension. Second, deficiency of Nox2 prevented hypertension-induced impairment of endothelium-dependent dilatation in cerebral arterioles.

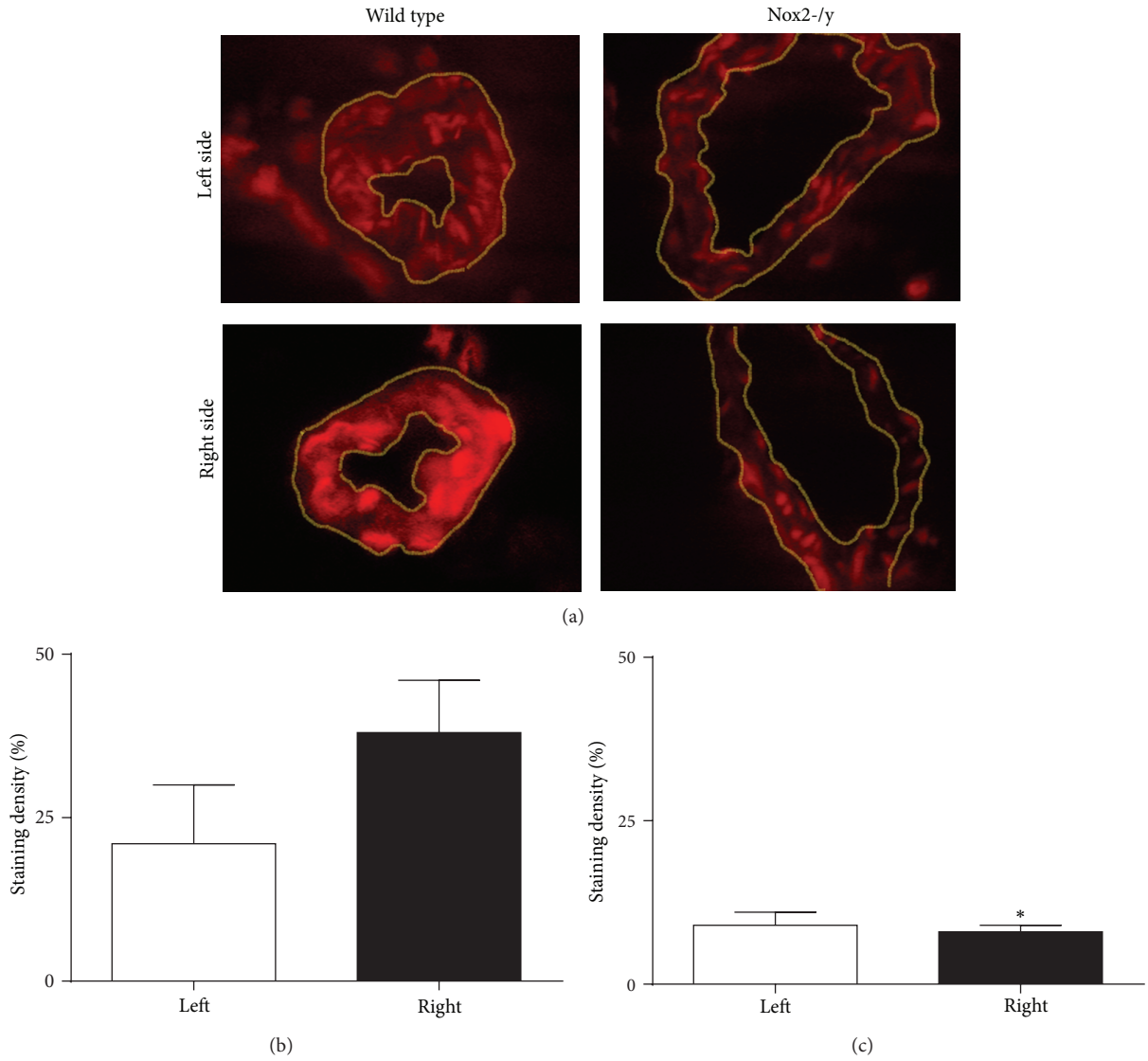

Figure 1: Representative micrographs of superoxide levels determined by hydroethidium fluorescence of WT left-sided, WT right-sided, Nox2-/y left-sided, and Nox2-/y right-sided cerebral arterioles (highlighted in yellow) (a). Graphs showing relative staining density of WT (b) and Nox2-/y mice (c) ($n = 8$). $^{*}P < 0.05$ versus WT right-sided cerebral arterioles.

Moreover, L-NAME treatment eliminated the normalized endothelial function in hypertensive cerebral arterioles of Nox2-/y mice, suggesting an NO-dependent mechanism. Third, hypertension caused hypertrophy in cerebral arterioles from WT mice, but not in Nox2-/y mice. This suggests that ROS derived from Nox2-containing NADPH oxidase play a key role in hypertension-induced hypertrophy in cerebral arterioles. Interestingly, Nox2 deficiency did not prevent hypertrophy in carotid artery. Taken together, this study provides *in vivo* evidence that chronic hypertension induces cerebral arteriolar dysfunction and hypertrophy via increased production of ROS derived from Nox2-containing NADPH oxidase.

We used ethidium fluorescence to examine the effects of Nox2 deficiency on hypertension-induced production of superoxide in cerebral arterioles. Being aware of potential problems with this method, matched pairs of hypertensive (right-sided) and normotensive (left-sided) cerebral arterioles from each mouse were examined in parallel using the same reagents and laser settings. In addition, we have shown previously that incubation with PEG-SOD, a scavenger of superoxide, abolishes ethidium fluorescence in aorta of mice that overexpresses human renin and human angiotensinogen [23].

Chronic hypertension is well known to increase vascular production of ROS. Using a model of abdominal aortic banding, it was shown previously that superoxide levels are elevated in noncerebral vessels [13, 14]. In the present study, transverse aortic banding was used to increase pressure in right-sided (hypertensive) cerebral arterioles relative to left-sided (normotensive) cerebral arterioles. We found in WT mice that the production of superoxide was elevated in

Table 1: Summary of blood pressures (measured by carotid catheters in anesthetized mice) and arterial blood gases in WT and Nox2-/y mice.

Parameters	WT		Nox2-/y		WT + L-NAME		Nox2-/y + L-NAME	
	Left	Right	Left	Right	Left	Right	Left	Right
Systemic arterial pressure (mm Hg)								
Systolic	70 ± 3	90 ± 4*	73 ± 2	95 ± 2*	67 ± 2	91 ± 4*	65 ± 2	84 ± 2*
Diastolic	55 ± 2	55 ± 2	63 ± 2	64 ± 2	52 ± 3	52 ± 3	52 ± 3	53 ± 3
Mean	62 ± 2	68 ± 3	67 ± 2	75 ± 2	58 ± 3	66 ± 3	57 ± 3	64 ± 2
Pulse	13 ± 2	32 ± 3*	9 ± 1	30 ± 3*	14 ± 2	38 ± 5*	12 ± 2	29 ± 3*
Arterial blood gases								
pH	7.39 ± 0.02		7.41 ± 0.01		7.35 ± 0.03		7.33 ± 0.03	
PCO_2	28 ± 2		27 ± 1		33 ± 3		33 ± 4	
PO_2	114 ± 9		113 ± 5		104 ± 6		108 ± 8	
Age (week)	12.9 ± 0.6		13.4 ± 0.2		14.7 ± 0.4		14.3 ± 0.7	
Weight (g)	23.1 ± 0.8		22.1 ± 0.5		22.2 ± 0.8		20.5 ± 1.1	
N	14		17		17		15	

* $P < 0.05$ versus left-sided cerebral arterioles.

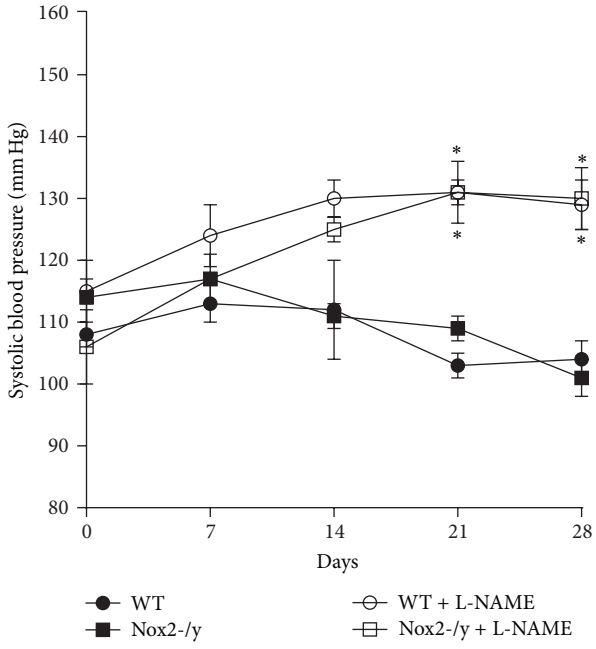

Figure 2: Graph showing systolic blood pressures measured by tail-cuff method. Results were the averages of thirty measurements made on days 0, 7, 14, and 28 of 6 individual mice. * $P < 0.05$ versus corresponding control treatment group.

right-sided cerebral arterioles relative to left-sided arterioles, whereas in Nox2-/y mice levels of superoxide were essentially the same in right- and left-sided arterioles. This finding indicates that Nox2-containing NADPH is the major source of increased production of ROS in cerebral arterioles during hypertension.

It is still debatable as to whether Nox2-derived ROS play a role in the development of hypertension. For example, the pressor response to angiotensin II was found to be reduced in Nox2-/y mice in one study [24] and unaffected in another [25]. The finding in this study that L-NAME increased systemic blood pressure in Nox2-/y mice to levels similar to those found in WT mice supports the idea that Nox2-derived ROS do not contribute significantly to hypertension.

Endothelial dysfunction caused by hypertension in the cerebral circulation has been previously demonstrated in various animal models [10, 26]. To our knowledge, this is the first study to examine endothelium-dependent function in cerebral arterioles using the transverse aortic banding model in mice. One of the advantages of this model is that the contralateral side can be used as a normotensive control. Previous studies found disparate effects of hypertension induced by aortic banding on endothelium-dependent function in aorta or coronary arteries [13, 27], probably due to the differences in the vascular beds studied and the location of the band. In this study, our finding that transverse aortic banding impairs endothelium-dependent dilatation in right-sided (hypertensive) cerebral arterioles in WT mice, but not in Nox2-/y mice, suggests that superoxide derived from Nox2-containing NADPH oxidase may play an important role in altered endothelium-dependent function in cerebral arterioles during hypertension.

Hypertension-induced impairment of endothelium-dependent dilatation is thought to result from reduced availability of NO due either to its destruction by NADPH-derived superoxide or to an uncoupling of eNOS, which results in the production of superoxide instead of NO. Our finding that Nox2 deficiency protected against impairment in cerebral arteriolar dilatation during hypertension produced with transverse aortic banding, but not L-NAME, supports the concept that hypertension impairs endothelial-dependent dilatation of cerebral arterioles through the destruction of NO by NADPH-derived superoxide, and not by uncoupling of eNOS.

Cerebral vascular hypertrophy is a well-known consequence of hypertension [17, 19, 21]. A role for superoxide in the development of vascular hypertrophy is implicated by the

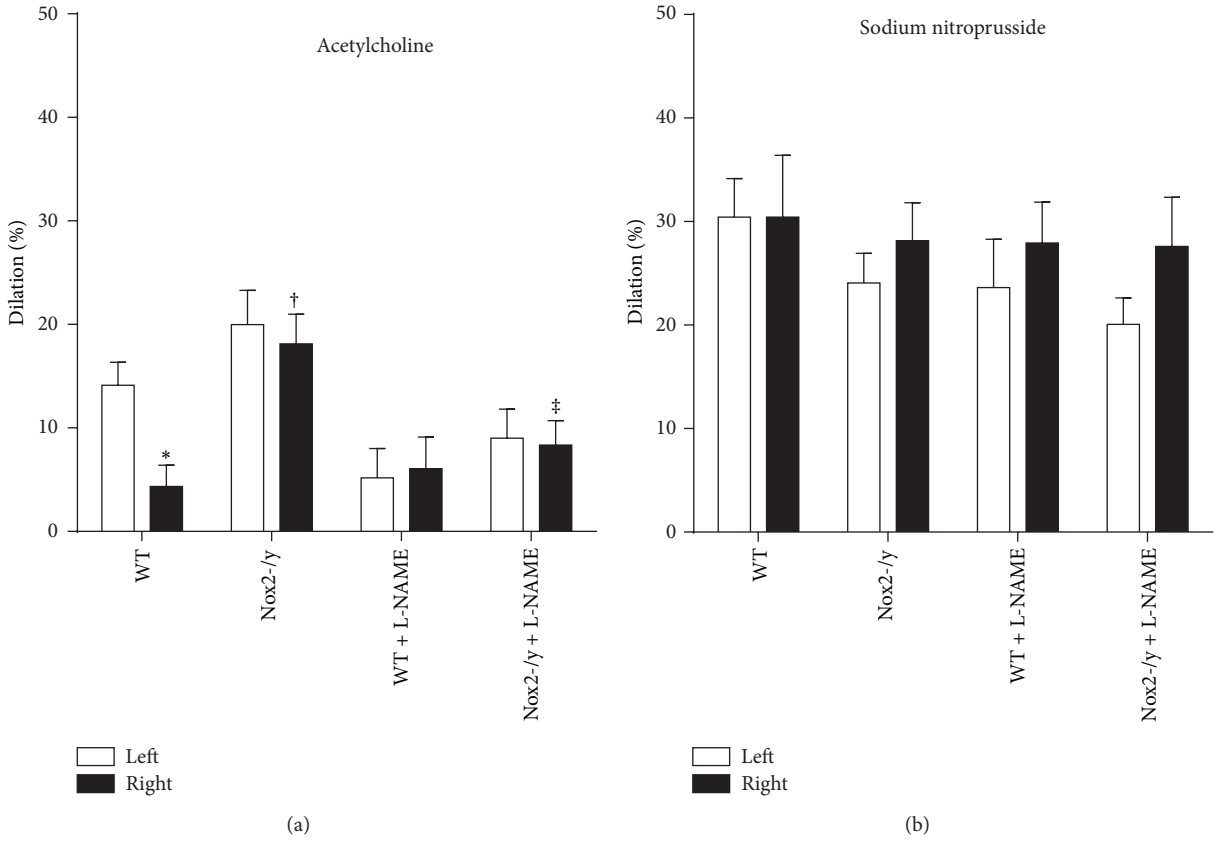

Figure 3: Graph showing endothelium-dependent and -independent vasodilation in cerebral arterioles. Acetylcholine (Ach, 10^{-5} mol/L) (a) or sodium nitroprusside (SNP, 10^{-7} mol/L) (b) was suffused in artificial CSF for 5 min in 8 mice. $^{*}P < 0.05$ versus left-sided WT group; $^{\dagger}P < 0.05$ versus right-sided WT group; and $^{\ddagger}P < 0.05$ versus right-sided Nox2-/y group.

finding that mice deficient in copper-zinc superoxide dismutase develop cerebral arteriolar hypertrophy while remaining normotensive [6]. Additional support for this concept is provided by the finding in this study that whereas transverse aortic banding resulted in hypertrophy of hypertensive cerebral arterioles in WT mice, hypertensive arterioles did not undergo hypertrophy in Nox2-/y mice.

One mechanism by which superoxide may promote vascular hypertrophy is through the destruction of NO [28]. NO has been shown to inhibit mitogenesis and proliferation of cultured smooth muscle [29], and treatment with L-NAME, as well as deficiency of eNOS, has been shown to induce hypertrophy of cerebral arterioles in mice [17]. Further support for this concept would appear to be provided by our finding in this study that treatment of aortic banded WT mice with L-NAME tended to cause hypertrophy in normotensive (left-sided), as well as hypertensive (right-sided), cerebral arterioles ($P = 0.063$ versus untreated WT mice). However, the possibility that destruction of NO may not be a critical factor in the development of ROS-induced hypertrophy of cerebral arterioles is suggested by our finding that the treatment of Nox2-dieficient mice with L-NAME did not induce hypertrophy in either hypertensive or normotensive cerebral arterioles. Instead, this finding suggests that ROS derived from Nox2-containing NADPH oxidase may play a central role in the development of cerebral arteriolar hypertrophy.

While we cannot exclude the possibility that ROS downstream of superoxide, such as peroxynitrite or hydrogen peroxide, contribute to the hypertrophic process, we believe that superoxide plays a more important and direct role in causing cerebral vascular hypertrophy. We base this speculation on two observations. First, we showed in this study that L-NAME inhibition, which supposedly limits the interaction of superoxide and NO to form peroxynitrite, does not attenuate the degree of cerebral arteriolar hypertrophy induced by transverse aortic banding in WT mice. Second, we showed in a previous study that the deficiency of copper-zinc superoxide dismutase, which leads to reduced conversion of superoxide to hydrogen peroxide, nevertheless causes hypertrophy in cerebral arterioles [6].

5. Conclusion

The present study demonstrated that ROS derived from Nox2-containing NADPH oxidase are critical in hypertension-mediated cerebral arteriolar vascular dysfunction and hypertrophy. This may lead to reduction of dilator capacity

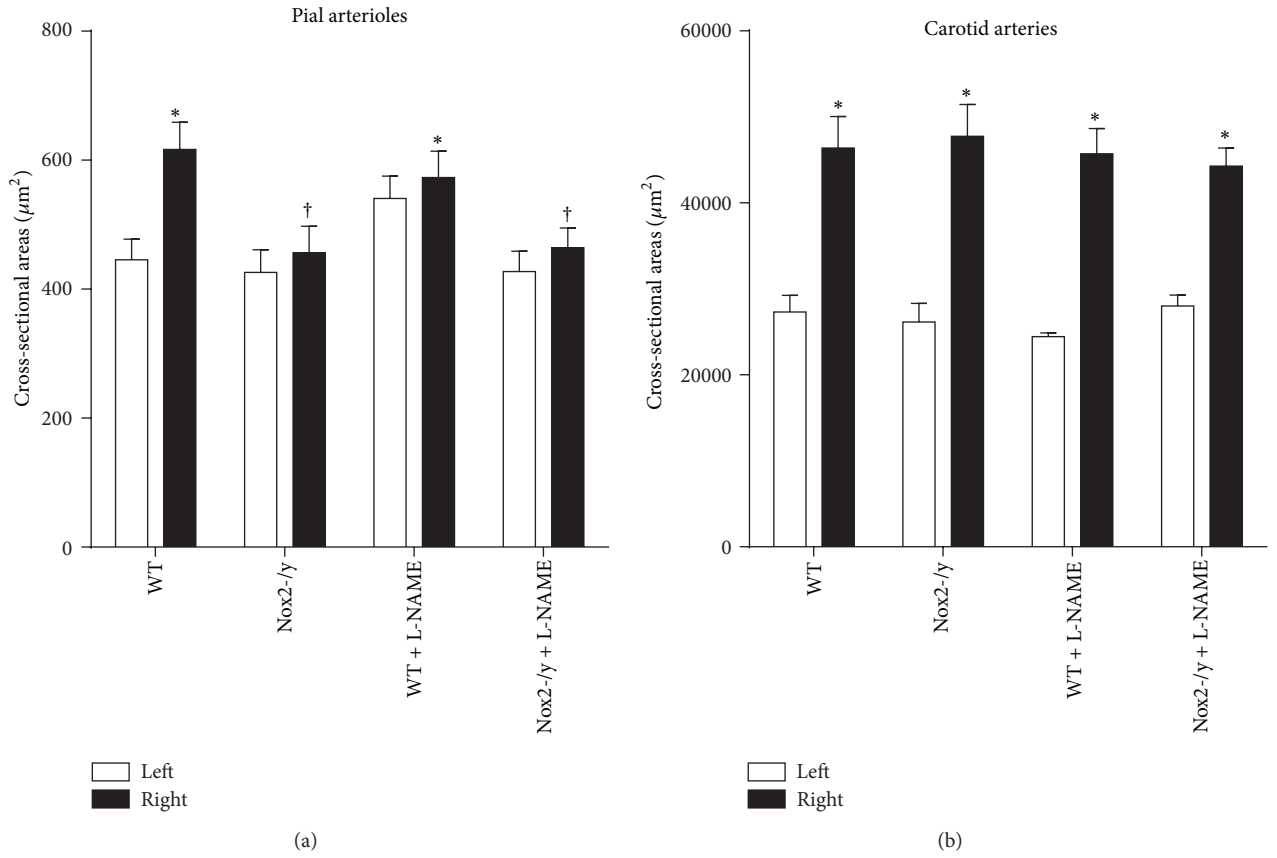

FIGURE 4: Graph showing cross-sectional areas of maximally dilated cerebral arterioles (a) and carotid arteries ($n = 8$) (b). $^{*}P < 0.05$ versus left-sided WT group; $^{\dagger}P < 0.05$ versus right-sided WT group.

and the ability to control local cerebral blood flow during hypertension.

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Research Article

Antioxidant Treatment Reverts Increased Arterial Basal Tone and Oxidative Stress in Nephrectomized (5/6) Hypertensive Rats

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Nonischemic 5/6 nephrectomized rat (NefR) is a model of chronic kidney disease. However, little is known about vascular dysfunction and its relation with hypertension in NefR. *Aims*. To evaluate possible alterations of endothelial function, NO-bioavailability, and basal tone in aorta from NefR and the role of oxidative stress. Sprague Dawley rats were divided into sham rats (SR), NefR, and NefR treated with tempol (NefR-T). Mean arterial pressure (MAP) and renal function were determined. In isolated aortic rings the following was measured: 1-endothelial function, 2-basal tone, 3-NO levels, 4-membrane potential (MP), and 5-oxidative stress. NefR increased MAP (SR: 119 ± 4 mmHg; $n = 7$; NefR: 169 ± 6; $n = 8$; $P < 0.001$). Tempol did not modify MAP (NefR-T: 168 ± 10; $n = 6$; $P < 0.001$). NefR showed endothelial dysfunction, increased basal tone and decreased NO levels (SR: 32 ± 2 nA; $n = 7$, NefR: 10 ± 2; $n = 8$; $P < 0.001$). In both in vitro and in vivo tempol improves basal tone, NO levels, and MP. Oxidative stress in NefR was reverted in NefR-T. We described, for the first time, that aorta from NefR presented increased basal tone related to endothelial dysfunction and decreased NO-bioavailability. The fact that tempol improves NO-contents and basal tone, without decrease MAP, indicates that oxidative stress could be implicated early and independently to hypertension, in the vascular alterations.

1. Introduction

It is known that, in the chronic kidney disease (CKD), the endothelial dysfunction could be cause and/or consequence of the kidney damage. Nonischemic 5/6 nephrectomized rat (NefR) is a model used to evaluate the evolution of renal abnormalities in CKD [1]. A lot of evidence has reported that the decrease of renal mass is a cardiovascular risk factor [2]. Recently the concept that reduced renal mass is associated with low birth weight as a cardiovascular risk factor has gained importance. These suggestions have been supported by both clinical [3] and experimental evidence [4]. Xie et al. showed that rats with low birth weight impaired renal function and developed hypertension.

In NefR, the impact of the loss of renal mass, its relationship to vascular dysfunction, and the development of hypertension have been little studied. It has been shown, in rats, that the renal mass reduction (2/3) associated with ischemia of contralateral kidney was able to induce hypertension [5]. In this situation, the hypertension has been explained by renin-angiotensin system activation. However, the implicated mechanisms in the development of hypertension in NefR without renal ischemia are unclear. In this model, to the best of our knowledge, arterial basal tone, endothelial function, and nitric oxide (NO), which are known to be involved in the regulation of arterial pressure have not been evaluated in a comprehensive way. In NefR, some authors did not find hypertension [6, 7]; however other authors found elevated

values of blood pressure [8]. Recently, Toba et al. [9] showed that NefR developed hypertension, which does not decrease with the administration of the substrate of NO synthase (NOS), L-arginine.

On the other hand, it is known that models of hypertension, like spontaneously hypertensive rats (SHR) [10], have alterations in basal tone of vascular smooth muscle cells (VSMC). Accordingly, we previously demonstrated that arterial vessels from SHR [11] and coarctation-hypertensive rats [12] showed increased basal tone. This increased basal tone was evidenced by a relaxant response to sodium nitroprusside (SNP) and atrial natriuretic peptide. Other findings from our laboratory, in isolated human arteries, showed an increased basal tone in both hypertensive and normotensive patients [13], indicating that the basal tone of VSMC is not only a consequence of the development of hypertension. Consequently, the integrity of the vascular function and the bioavailability of NO could play a pivotal role in vascular homeostasis and its alteration is involved in an increased basal tone. Another factor that could alter the basal tone is the oxidative stress [14] through inactivation of NO and alteration of vascular reactivity. Moreover, the oxidative stress may also produce a direct vasoconstrictor effect [15] accompanied to an increase of myogenic tone. NADPH oxidase is a source of superoxide anion in the vessel wall. Superoxide anion is involved in increased blood pressure, vascular hypertrophy, and endothelial dysfunction [15, 16] and plays a role in the development of spontaneous vascular tone [11].

In the light of these considerations, the objectives of the present work were to evaluate possible alterations of the endothelial function, NO bioavailability, and basal tone in aortic rings from NefR and establish the role of oxidative stress.

2. Methods

2.1. Animal Model. In order to obtain a nonischemic model of renal mass reduction, two surgeries were performed to male Sprague Dawley rats: first, the resection of 2/3 of the left kidney (the two poles) and second, after 2 weeks, a complete resection of the right kidney. After each surgery the animals were placed in acclimatized cages until recovery. After 13-14 weeks of second surgery, creatinine clearance (Ccr) (Wiener Lab, Argentina) and microalbuminuria (BioSystem kit, Spain) were measured in metabolic cage by 24 hours. Mean arterial pressure (MAP) was measured by direct method through cannulation of right carotid with a catheter connected to a pressure transductor [12]. In all surgical procedures the animals were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneal). In all cases the rats were sacrificed under anesthesia by exsanguination. The thoracic aorta was dissected, immersed in Krebs solution (in mmol/L: NaCl 122, KCl 5.9, $NaHCO_3$ 25, $CaCl_2$ 1.9, $MgSO_4$ 1.2, and glucose 11), and cut into 5 mm rings (1 to 4 for rat). In some experiments the endothelium was removed (rubbed rings). The rats were divided into 2 groups: one without treatment (NefR, $n = 8$) and other treated with tempol (1 mmol/L in drinking water) since the first surgery until sacrifice (NefR-T, $n = 6$). Results were compared with sham rats (SR: $n = 7$). In SR the two surgeries were performed without removal of any portion of the renal mass.

All experiments were carried out according to the guidelines of the institutional ethics committee.

2.2. Contractility. The rings were fixed in an isolated organ chamber with 6 mL of Krebs solution maintained at 37°C, gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4) [13], and connected to a force transducer (GOULD UC2, USA) and to a recorder (K&Z BD41, Holland). The rings were equilibrated at 2 g of tension by 120 min, which was found to be the optimal tension for KCl-induced contraction (100 mM). KCl-contraction was similar in all groups (SR: 1392 ± 150 mg; $n = 7$; NefR: 1187 ± 131; $n = 8$ and NefR-T: 1028 ± 154; $n = 6$; P: NS). Data were expressed in milligrams (mg) of tension.

To evaluate the spontaneous basal tone, the endothelium-independent agent (SNP) was added to rings not previously exposed to any vasoactive agent [12]. For this purpose, SNP was used to maximal dose (10^{-5} M), which was found to be the optimal concentration for SNP-induced relaxation (100%) of the precontracted rings with norepinephrine (NE).

In some rings, SNP-response was evaluated after incubation (20 min) with Nω-nitro-L-arginine methyl ester (L-NAME) 10^{-4} M.

In NefR, in order to evaluate the possible role of oxidative stress in the basal tone, unrubbed aortic rings were subjected to a "preconditioning manoeuvre." For this purpose some aortic rings were incubated with tempol 10^{-5} M or diphenyliodonium (DPI) 10^{-4} M during 120 min (preconditioned vessels). After that, this agent was removed by washing (40 min) with Krebs solution and SNP stimulation was performed.

Endothelial function was tested with cumulative dose response curve to acetylcholine (Ach) (10^{-9}–10^{-5} M) in NE 10^{-5} M-contracted rings. In all cases the maximal relaxation was induced by Ach 10^{-5} M. Control experiments were performed in rings incubated (20 min) with L-NAME 10^{-4} M.

2.3. Nitrites. Nitrites were measured, by the Griess reaction, in samples from the bath of isolated aortic rings subjected to stretching (2 g) [13]. In some rings, nitrites were measured in the presence (incubation by 20 min) of L-NAME 10^{-4} M, tempol 10^{-5} M, or DPI 10^{-4} M. Data were expressed in pmol/mg of tissue.

2.4. Direct NO Measurement. NO release was measured in real time with an ISO-NOP electrode (WPI, USA) connected to a recorder (Apollo 4000, WPI, USA) in isolated aortic rings subjected to stretching [17] at optimal tension for KCl-induced contraction (2 g). Some rings were treated with L-NAME 10^{-4} M or tempol 10^{-5} M. Data were expressed in nanoamperes (nA).

2.5. Electrophysiological Studies. Membrane potential (MP) was recorded in VSMC with electrodes connected to an

Table 1: Clinical characteristics of rats.

	Rat		
	SR ($n = 7$)	NefR ($n = 8$)	NefR-T ($n = 6$)
Body weight (g)	226.3 ± 8.4	218.5 ± 4.5	220.5 ± 5.2
Food intake (g/24 hs)	24.7 ± 3.8	18.2 ± 1.8	23.0 ± 3.6
Water intake (mL/24 hs)	22.7 ± 1.6	$41.6 \pm 4.1^{***}$	$40.8 \pm 2.6^{\dagger\dagger\dagger}$
Urinary volume (mL/24 hs)	10.7 ± 2.2	$28.1 \pm 3.7^{***}$	$23.5 \pm 2.4^{++}$
Glucose (mg/dL)	80.0 ± 0.1	83.2 ± 1.3	82.1 ± 1.1
Mean arterial pressure (mm Hg)	119 ± 4	$169 \pm 6^{***}$	$168 \pm 10^{\dagger\dagger\dagger}$
Heart rate (beats/min)	365 ± 21	353 ± 16	354 ± 15
Creatinine clearance (mL/min)	0.59 ± 0.1	$0.11 \pm 0.01^{***}$	$0.22 \pm 0.04^{\dagger\dagger,+}$
Microalbuminuria (mg/24 hs)	6.8 ± 1.2	$62.9 \pm 9.8^{***}$	$22.1 \pm 5.2^{\dagger,+++}$

SR: sham rats; NefR: nefrectomized rats; NefR-T: NefR treated with tempol; $^{***}P < 0.001$ NefR versus SR; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ NefR-T versus SR; $^{+}P < 0.05$, $^{++}P < 0.01$, and $^{+++}P < 0.001$ NefR-T versus NefR. One way ANOVA.

amplifier (WPI, USA) and to a recorder (Gould, USA) [18] in basal conditions and after in vitro treatment with tempol 10^{-5} M. Results were expressed in mV as differences between MP obtained by KCl 100 mM (SR: +7.0 ± 2.7 mV; $n = 6$; NefR: +6.0 ± 1.7; $n = 6$; NefR-T: +6.6 ± 1.2; P: NS) and MP registered (basal or tempol).

2.6. Oxidative Stress. Total, reduced (GSH), and oxidized glutathione (GSSG) and thiobarbituric acid reactive substances (TBARS) were determined in aorta homogenates by spectrophotometry [17, 19, 20] and correlated with protein contents.

2.7. Statistical Analyses. Results were expressed in mean ± standard error. Statistical analyses were performed with Statistica 5.0 programs. Student's t-test for paired samples and ANOVA (One or Two way with Newman-Keuls posttest) were used when appropriate. Results were considered significant when $P < 0.05$.

3. Results

Clinical characteristics of the animals are shown in Table 1. Resection of 5/6 of renal mass (NefR) induces an increase of MAP and microalbuminuria, whereas Ccr was decreased. In vivo treatment with tempol (NefR-T) was ineffective in reversing the hypertension but was able to improve Ccr and microalbuminuria (Table 1). Urinary nitrites were higher in SR (417.6 ± 59.2 pmol/mL; $n = 6$) than NefR (94.5 ± 18.5; $n = 6$; $P < 0.001$; One way ANOVA) and NefR-T (227.5 ± 49.0 pmol/mL; $n = 6$; $P < 0.01$; One way ANOVA). NefR-T improves urinary nitrites in respect to NefR ($P < 0.05$; One way ANOVA).

3.1. Endothelial Function. Endothelial function, checked through Ach-vasorelaxant response, was present in SR aortic rings: −796 ± 187 mg (−70 ± 18% of NE-contraction, $n = 7$). A significant decrease of Ach-response was observed in NefR: −70 ± 10 mg (−30 ± 4% of NE-contraction, $n = 8$; $P < 0.01$ in respect to SR; One way ANOVA). NefR-T improves endothelial function: the Ach-vasorelaxation was −312 ± 169 (−50 ± 6% of NE-contraction, $n = 6$; $P < 0.05$ in respect to NefR; One way ANOVA). There were not significant differences in Ach-response between SR and NefR-T (P: NS; One way ANOVA). In all cases, rubbed manoeuvres or incubation with L-NAME abolished the Ach-response (data not shown).

3.2. Basal Tone. Figure 1(a) shows recorders from typical experiments of the effect of SNP on the basal tone. SNP had no effect on aortic rings from SR (upper panel). However, SNP produced a vasorelaxant response in aortic rings from NefR (middle panel), indicating an increased basal tone. In vivo treatment with tempol (NefR-T) reverted near completely the SNP effect (down panel), indicating a decreased basal tone. The averages of these responses are shown in Figure 1(b) (first group of bars). In any case the rubbed manoeuvres did not modify the effect of SNP (Figure 1(b), second group of bars).

In vitro incubation with L-NAME did not modify SNP-response on basal tone in aortic rings from SR (Krebs: −41 ± 15 mg, $n = 6$ versus L-NAME: −38 ± 15, $n = 6$; P: NS), NefR (Krebs: −1052 ± 149 mg, $n = 8$ versus L-NAME: −967 ± 78, $n = 6$; P: NS), and NefR-T (Krebs: −168 ± 44 mg, $n = 6$ versus L-NAME: −206 ± 22, $n = 6$; P: NS).

On the other hand, in aortic rings from NefR, in vitro preconditioning manoeuvre with antioxidant agents was effective to reverse the increased basal tone. Preconditioning with tempol (10^{-5} M by 120 min) decreased the SNP response. The effect was −244 ± 83 mg (−77 ± 8% from baseline, $n = 6$; $P < 0.01$). Similar effect was obtained by preconditioning with DPI (10^{-4} M by 120 min): −87 ± 3% from baseline; $n = 6$; $P < 0.01$.

3.3. Nitrite Levels. Figure 2 shows nitrite levels in aortic rings. Nitrite levels were higher in SR than NefR. NefR-T improves the nitrite levels. In all cases rubbing manoeuvres decreased nitrites (Figure 2).

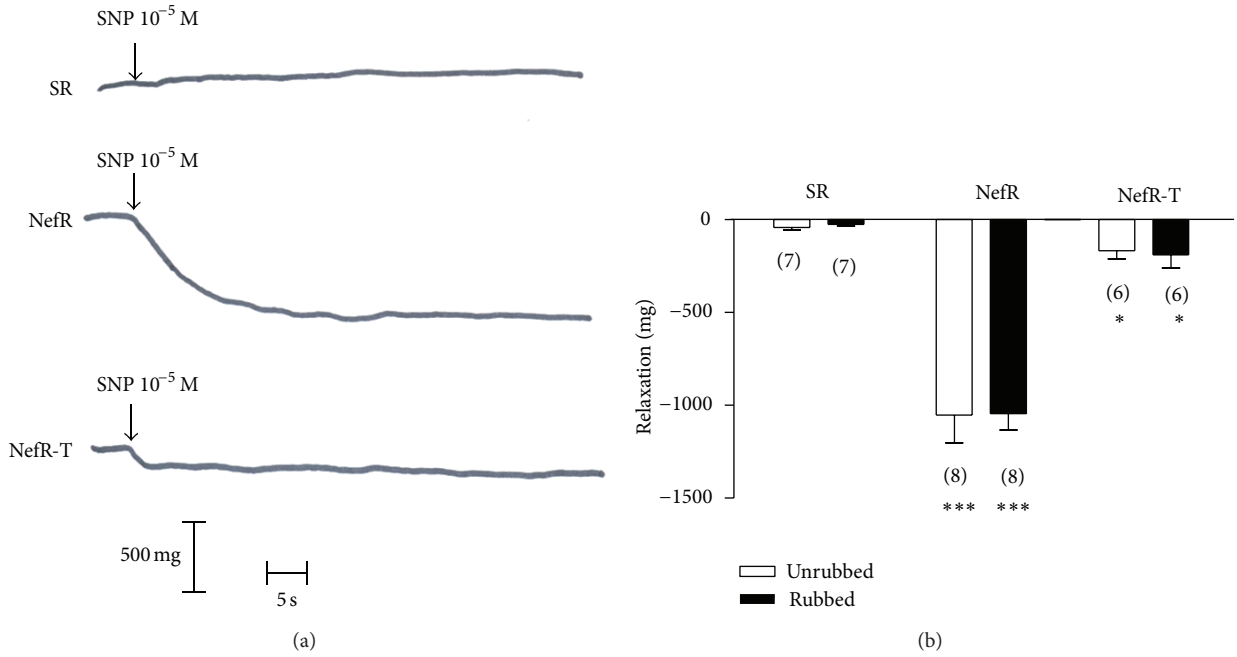

Figure 1: Effect of SNP 10^{-5} M on basal tone. (a) Tracing of a typical experiment of administration of SNP (arrows) on basal tone in unrubbed aortic ring from SR (upper), NefR, (middle) and NefR-T (lower). (b) Average of effect of SNP on basal tone in unrubbed (white bars) and rubbed (black bars) aortic rings from SR, NefR, and NefR-T. $^{***}P < 0.001$ NefR versus SR; $^{*}P < 0.05$ NefR-T versus SR. Two way ANOVA. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

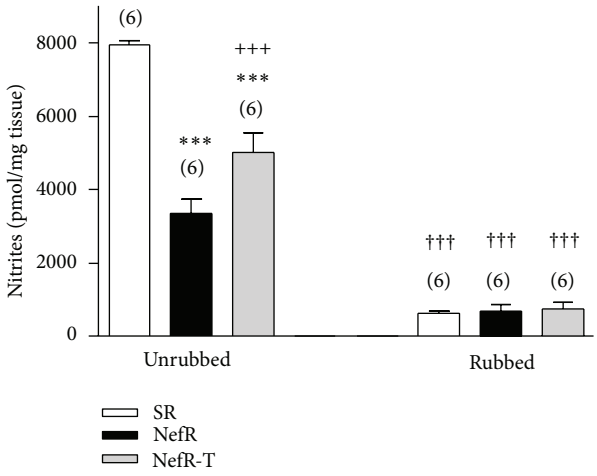

Figure 2: Nitrite contents in aortic rings of SR (white bars), NefR (black bars), and NefR-T (gray bars) with (first group of bars: unrubbed) or without functional endothelium (second group of bars: rubbed) at 15 min in the experiment. $^{***}P < 0.001$ NefR and NefR-T versus SR; $^{+++}P < 0.001$ NefR-T versus NefR; $^{\dagger\dagger\dagger}P < 0.001$ rubbed versus unrubbed rings. Two way ANOVA. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

In the presence of L-NAME 10^{-4} M, nitrite levels were decreased in unrubbed rings from SR (−90 ± 2% from baseline; $n = 6$; $P < 0.001$; paired Student's t-test), NefR (−75 ± 10% from baseline; $n = 6$; $P < 0.001$; paired Student's t-test), and NefR-T (−92 ± 5% from baseline; $n = 6$; $P < 0.001$; paired Student's t-test).

In NefR aortic rings, in vitro administration of tempol increased nitrite levels. The nitrite values in the presence of tempol 10^{-5} M were 6369 ± 433 pmol/mg ($n = 6$; 89 ± 13% increase over baseline; $P < 0.01$; paired Student's t-test). Similar results were observed with in vitro administration of DPI 10^{-4} M (73 ± 14% over baseline, $n = 7$; $P < 0.01$; paired Student's t-test). However, in SR and NefR-T these agents did not modify nitrites.

Figure 3 shows direct NO measurement in SR, NefR, and NefR-T and the effect of in vitro administration of tempol. Figure 3(a) shows recorders from typical experiments of the effect of tempol on NO release in SR (upper panel), NefR (middle panel), and NefR-T (bottom panel). Figure 3(b) shows the average of these responses. First group of bars shows the NO levels in basal conditions. NO release was higher in SR than NefR. NefR-T improves the NO release. Tempol had no effect in SR and NefR-T; however it was able to increase NO in NefR (Figure 3(b), second group of bars).

In vitro administration of L-NAME was able to decrease NO release in SR (−87 ± 1% from baseline; $n = 6$; $P < 0.001$; paired Student's t-test) and NefR (−64 ± 1% from baseline; $n = 8$; $P < 0.01$; paired Student's t-test). Similar inhibition was obtained in NefR-T (data not shown).

3.4. Membrane Potential. Figure 4 shows the MP in basal conditions and after administration of L-NAME in unrubbed aortic rings from SR, NefR, and NefR-T. Basal MP was higher in NefR than SR. NefR-T partially recovered MP values

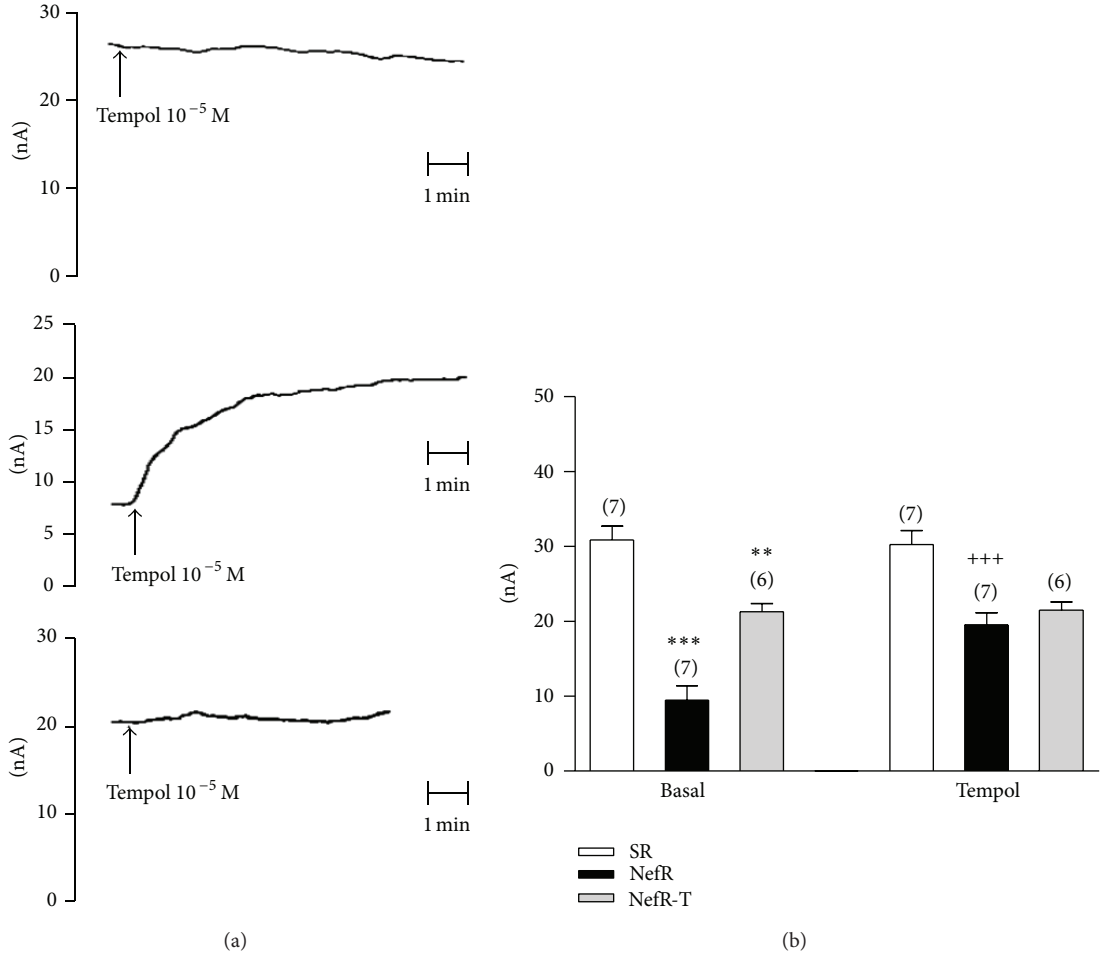

Figure 3: Effect of tempol 10^{-5} M on NO levels (a) Typical experiment of direct measurement of NO in unrubbed aortic rings from SR (upper), NefR (middle), and NefR-T (lower) and the effect of tempol (arrows). (b) Average of effect of tempol on direct measurement of NO in unrubbed aortic rings of SR (white bars), NefR (black bars), and NefR-T (gray bars). $^{**}P < 0.01$ NefR-T versus SR; $^{***}P < 0.001$ NefR versus SR; $^{+++}P < 0.001$ tempol versus basal. Two way ANOVA. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

(Figure 4, first group of bars). In vitro treatment with L-NAME produced a partial depolarization in SR and NefR-T but not in NefR (Figure 4, second group of bars).

In vitro administration of tempol induced a hyperpolarizing effect only in NefR (MP: −30 ± 1 mV; 124 ± 5% from baseline; $n = 6$; $P < 0.01$ paired Student's t-test).

3.5. Oxidative Stress. In aortic rings, GSH levels were higher in SR (33.9 ± 7.2 μmol/mg protein; $n = 6$) than NefR (3.8 ± 1.4; $n = 6$; $P < 0.001$). However, similar GSSG levels were found in SR (5.6 ± 0.6 μmol/mg protein; $n = 6$) and NefR (5.2 ± 0.3; $n = 6$; P: NS). In agreement, the GSH/GSSG ratio was higher in SR (6.3 ± 1.0; $n = 6$) than NefR (0.7 ± 0.2; $n = 6$; $P < 0.001$; One way ANOVA). NefR-T improves GSH/GSSG (data not shown).

In aortic rings, TBARS levels were higher in NefR (5.2 ± 0.7 nmol/mg protein; $n = 6$) than SR rings (1.5 ± 0.3; $n = 6$; $P < 0.001$; One way ANOVA). NefR-T decreased the TBARS levels (1.2 ± 0.6 nmol/mg protein; $n = 6$; $P < 0.001$ versus NefR; One way ANOVA). No significant differences were found between SR and NefR-T in TBARS levels (P: NS; One way ANOVA).

4. Discussion

The novel finding of this study is that (1) subtotal nephrectomy induces an associated hypertension with an increased basal tone, an endothelial dysfunction, and an oxidative state and that (2) in vivo treatment with tempol improves arterial basal tone, NO levels, and oxidative stress without reversing hypertension, indicating that oxidative stress could be implicated in the vascular alterations, and that these disorders would occur early and independently to hypertension.

It has been demonstrated that several models of renal mass reduction induce CKD and alter the renal function [6, 21, 22]. On the other hand, CKD is frequently associated with hypertension [23, 24]. A lot of evidence has reported that the decreased renal mass is a cardiovascular risk factor

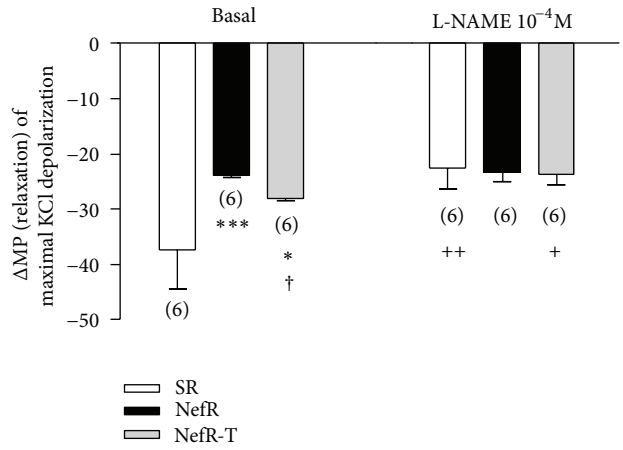

Figure 4: Membrane potential (MP) in unrubbed aortic rings from SR (white bars), NefR (black bars), and NefR-T (gray bars) in basal conditions (Krebs) and the effect of administration of L-NAME 10^{-4} M. $^{***}P < 0.001$ NefR basal versus SR basal; $^{*}P < 0.05$ NefR-T basal versus SR basal; $^{++}$SR L-NAME $P < 0.01$ versus SR basal; $^{+}P < 0.05$ NefR-T L-NAME versus NefR-T basal; $^{\dagger}P < 0.05$ NefR-T basal versus NefR basal. Two way ANOVA. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

[2]. In experimental models, some authors have reported that the loss of renal mass induces hemodynamic alterations that lead to hypertension [4]; however the physiopathological mechanisms involved remain unclear. It is known that an endothelial function and an NO bioavailability play a pivotal role in the balance of vascular tone. Likewise, an increase in basal tone may be associated with the presence of oxidative stress. In a model of CKD with loss of renal mass associated with renal ischemia, it has been shown that oxidative stress is implicated in the hypertension developed [25]. In our work, nonischemic 5/6 nephrectomized rats showed hypertension associated with an impaired endothelial function and a decreased NO levels. In agreement with this, other authors found that the vessels from nonischemic 5/6 nephrectomized rats have a decreased Ach-response indicating an endothelial dysfunction [26]; however, these alterations were developed in the absence of hypertension.

In relation to the increased basal tone in NefR, previous reports from our laboratory have shown an increased basal tone in coarctation-hypertensive rats [12] and SHR [11]. Also, we were able to increase the basal tone by an "in vitro" sensitizing manoeuvre in vessels from rabbits [27] and toads [28]. For this purpose, normotensive aortic rings were previously sensitized with a vasoconstrictor agent, washed with Krebs by 120 min, and then stimulated with a vasorelaxant agent. On the other hand, it is known that oxidative stress could alter the basal tone. In this sense, in our laboratory, we found that the basal tone of SHR was increased by oxidative stress [11]. Also, it was reported that the oxidant agent H_2O_2 could alter vascular contractility in normotensive and hypertensive rats [29]. This is in agreement with the finding from a present report, in which antioxidant treatment decreased the basal tone in aortic rings from NefR. An other result from present work that supports the fact that vessels of NefR presented an increased oxidative stress is the altered GSH/GSSG ratio and increased TBARS levels.

Despite that NefR and NefR-T showed similar values of MAP, the aortic rings from NefR-T decreased basal tone, evidenced by a lower SNP response. This fact could indicate that in vivo treatment with antioxidant agents may decrease basal tone independently of the blood pressure values. At variance with our findings, in other model of CKD associated with renal ischemia [25], tempol decreased MAP values. This difference may be explained since renal ischemia is not present in our model of CKD and, it is known that renal ischemia is strongly associated with great activation of renin-angiotensin system in which angiotensin II (Ang II) not only has hemodynamic effects but also has tissue actions, which results in an increase of oxidative stress. Accordingly, some works have demonstrated that Ang II activation of NADPH increases reactive oxygen species [30]. However, in our model these additional effects of Ang II on oxidative stress are not present.

The role of endothelium in the vascular function and its association with CKD are well known. In present work we found that NefR showed an endothelial dysfunction with decreased NO levels. The fact that in vivo treatment with tempol improves endothelial function and NO bioavailability indicates that the endothelium damage is produced, at least in part, by oxidative stress.

In our work we found that NefR decreased urinary nitrites. Similar findings have been reported by other authors, who also found reduced renal NO-synthases in a similar model of subtotal nephrectomy [7]. Unlike other works performed in models of subtotal nephrectomy, in our study we measured nitrite and NO in vessels. We observed that the decreased vascular NO levels were increased by tempol (in vivo and in vitro), indicating a role of oxidative stress in the vascular NO bioavailability. In accordance, it has been reported that superoxide dismutase improves NO-dependent vasorelaxation in other models of CKD [25].

Assuming that the oxidative stress decreases NO levels and increases the basal tone [11], we could hypothesize that antioxidant treatment improves NO bioavailability and also decreases basal tone. In fact, in vitro administration of tempol improves NO bioavailability and reduces the SNP response in aortic rings. Moreover, the in vivo treatment with antioxidant agents (NefR-T) showed similar results.

In conclusion, in the present work we described, for the first time, that aortic rings from nonischemic 5/6 nephrectomized rats presented an increased basal tone related to endothelial dysfunction and decreased NO levels. The fact that in vitro and in vivo treatments with tempol improve the NO bioavailability and the basal tone, without decreasing the values of blood pressure, indicates that oxidative stress could be implicated in the vascular alterations and that these disorders would occur early and independently to hypertension.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

DNA Damage and Augmented Oxidative Stress in Bone Marrow Mononuclear Cells from Angiotensin-Dependent Hypertensive Mice

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It has been proposed that the nonhemodynamic effects of angiotensin II are important for the damage observed in the two-kidney, one-clip (2K1C) renovascular hypertension model. Much evidence confirms that angiotensin II is directly involved in NAD(P)H oxidase activation and consequent superoxide anion production, which can damage DNA. The current study was performed to examine the effects of angiotensin-II-dependent hypertension in bone marrow mononuclear cells (BM-MNC); dihydroethidium staining was used to assess reactive oxygen species (ROS) production, and the comet assay was used to assess DNA fragmentation in 2K1C hypertensive mice 14 days after renal artery clipping. In this study we demonstrated that 2K1C hypertensive mice have an elevated lymphocyte count, while undifferentiated BM-MNC counts were diminished. 2K1C mice also showed an augmented ROS production and marked BM-MNC DNA fragmentation. In conclusion, endogenous renin angiotensin system activation-induced arterial hypertension is characterized by excessive ROS production in BM-MNC, which might cause marked DNA damage.

1. Introduction

High blood pressure is commonly found in patients with chronic kidney disease and renovascular hypertension is a common form of secondary hypertension and frequently resistant to pharmacologic treatment [1]. In the two-kidney, one clip (2K1C) Goldblatt model, renovascular hypertension is induced by unilateral renal artery stenosis, which reduces renal perfusion of the clipped kidney and causes increased renin release and circulating angiotensin II (Ang II) [2]. Ang II, which is the main effector peptide of the renin-angiotensin system (RAS), has marked hemodynamic, cardiac, and renal effects, as previously observed by our laboratory in mice [2–5]. In addition, it also exerts tissue-specific responses as it can be locally synthesized [6–8]. Although it is controversial, the existence of a local bone marrow (BM) RAS has been demonstrated in rats [9]. Because the BM is a highly organized, complex organ, that is, the principal hematopoietic tissue in adults, locally BM-formed Ang II may be an autocrine or paracrine peptide that affects physiological and pathological hematopoiesis [10].

Studies have demonstrated that Ang II plays a role in oxidative stress development in the spontaneously hypertensive rat [11] and in the renovascular hypertensive rat [12]. Reactive oxygen species (ROS) play a crucial role in RAS signaling in BM cells [9, 13]. In addition, studies in experimental animals have shown that augmented ROS [14–16], particularly superoxide ($\bullet O_2^-$) [17–20], can interact

with DNA, which results in oxidative damage and DNA fragmentation-mediated cellular injury [21].

Taken together, this evidence strongly supports the importance of the 2K1C murine experimental model to investigate the influence of hypertension on DNA damage. Therefore, in the present study, we tested the hypothesis that 2K1C-mediated hypertension increases ROS production and induces DNA damage in murine BM mononuclear cells (MNC).

2. Material and Methods

2.1. Animals. Experiments were performed in male C57BL/6 (C57) mice, which present a single renin gene [22], weighing 23 g on average, and that were bred and maintained in the Laboratory of Transgenes and Cardiovascular Control animal facility (Vitoria, ES, Brazil). The mice were fed a standard chow diet and provided water *ad libitum*. Animals were housed in individual plastic cages with controlled temperature (22°C) and humidity (60%) and were exposed to a 12 : 12 h light-dark cycle. All of the experimental procedures were performed in accordance with the National Institutes of Health (NIH) guidelines, and study protocols were previously approved by the Institutional Animal Care and Use Committee (CEUA-Emescam Protocol no. 010/2009).

2.2. Induction of 2K1C Renovascular Hypertension. We used a mouse model of 2K1C angiotensin-dependent hypertension, as previously described [3, 4, 23]. Briefly, the animals were anesthetized (ketamine/xylazine 91/9.1 mg/kg, *i.p.*) and kept on a heating pad that maintained the body temperature at 37°C to avoid hypothermia. The left renal artery was exposed through a retroperitoneal flank incision and was carefully isolated from the renal vein, nerves, and connective tissues. A U-shaped stainless steel clip with a 0.12 mm opening width was placed around the renal artery close to the abdominal aorta, which resulted in partial renal perfusion occlusion [24]. The wound was sutured, and the animal received a single injection of benzylpenicillin benzathine (7 mg/kg, *i.m.*) followed by recovery under care for 24 h. Control mice underwent the same surgical procedure except for the renal artery clip placement (Sham).

2.3. Hemodynamic Measurements. Fourteen days after the renal artery clipping (2K1C) or Sham operations, the animals were anesthetized with a combination of ketamine/xylazine (91/9.1 mg/kg, *i.p.*) and the right common carotid artery was exposed and isolated through a cervical incision. A catheter (0.040 mm OD × 0.025 mm ID; Micro-Renathane; Braintree Scientific) was filled with heparin solution (50 UI/mL saline) and prior to insertion into the right carotid artery, which was subcutaneously tunneled and brought out at the nape of the neck. Immediately after surgery, animals received a single benzylpenicillin benzathine (7 mg/kg, *i.m.*) injection. The catheter was connected to a pressure transducer (Cobe Laboratories, USA), which was plugged into a pressure-processor amplifier and data acquisition system (MP100, Biopac Systems, USA) for mean arterial pressure (MAP) and heart rate (HR) recordings. After 48 hours, MAP and HR direct recordings were obtained while the animals were conscious and moving around freely in their cage.

2.4. Plasma Ang II Level Measurement. After hemodynamic measurements, blood was drawn through the arterial line into tubes containing EDTA and protease inhibitor cocktail (Product no. P2714, Sigma-Aldrich); the samples were centrifuged at 9.5 g for 15 min in a refrigerated centrifuge (4°C) to remove plasma for later analysis. Plasma Ang II was quantified by reverse phase high-performance liquid chromatography (HPLC). Briefly, peptides were initially separated in a reverse phase Aquapore ODS 300 column 7 μm (4.6 × 250 mm) (Applied Biosciences, Foster City, CA, USA) using a linear mobile phase gradient from 5 to 35% (acetonitrile in 0.1% phosphoric acid) for 40 min using a 1.5 mL/min flow rate. Ang III (320 ng) was added to each sample as an internal standard, and the peptides were detected at 214 nm absorbance. Ang II was extracted using Sep-Pack-C18 column chromatography (Millipore, MA, USA) and was activated with 5 mL methanol, 5 mL tetrahydrofuran, 5 mL hexane, and 10 mL H_2O (MilliQ). After activation, the samples were run through the column and eluted with ethanol : acetic acid : H_2O (90 : 4 : 6, v/v). The last phase eluate containing Ang II was evaporated in a Speed Vac SC 110 (Savant Instruments, Holbrook, NY, USA) and reconstituted with 500 μL 0.1% phosphoric acid in 5% acetonitrile, filtered, and injected onto the HPLC analytical column. Retention time was used to identify peaks of interest, which had been previously determined by standard peptide elution. The calculations were based on peak area, and Ang II concentration was expressed as pmol/mL blood.

2.5. Bone Marrow Mononuclear Cell Isolation. Mice were euthanized with a sodium thiopental overdose (100 mg/kg, *i.p.*) and marrow samples were collected from femurs and tibias that had been dissected and cleaned of all soft tissues. After removing the epiphyses and gaining access to the marrow cavities, whole BM was flushed out using a 26-gauge needle attached to a 1 mL syringe filled with Dulbecco's Modified Eagle Medium (DMEM; Sigma, St. Louis, MO, USA). MNCs were isolated by density-gradient centrifugation; the BM suspension in 4 mL DMEM was loaded on 4 mL Histopaque 1083 (Sigma-Aldrich) and centrifuged for 30 min at 400 g. The BM-MNC fraction was subsequently collected and washed in phosphate-buffered saline (PBS). A small volume of the resulting suspension was mixed with 0.4% trypan blue to perform cell count and viability analysis. Lymphocytes and undifferentiated cells were analyzed using a Neubauer chamber.

2.6. DNA Damage Measurement with the Comet Assay. Bone marrow MNC DNA damage was analyzed by the alkaline comet assay as described by Singh et al. [25] with minor modifications. Regular microscope slides were precoated with 200 μL 1.5% normal melting point agarose in distilled water, at 60°C (Sigma-Aldrich), dried overnight at room temperature, and then stored at 4°C until use. Subsequently, 2×10^4 MNCs were mixed with 100 μL 1% low melting point agarose in PBS at 37°C (Invitrogen, Spain) and spread on

the agarose-coated slides using a coverslip. After gelling at 4°C for 20 min, coverslips were removed, and the slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris at pH 10–10.5, with freshly added 1% Triton X-100 and 10% DMSO) at 4°C for 1 h. After a 5 min wash in cold distilled water, the slides were placed in an electrophoresis chamber, which was then filled with fresh alkaline buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4°C. Electrophoresis was performed at 300 mA and 25 V for 30 min. All of these steps were conducted without direct light to prevent additional DNA damage. The slides were washed three times for 5 min with 0.4 M Tris buffer, pH 7.5, for neutralization. Finally, 100 μL 20 μg/mL of ethidium bromide (Sigma-Aldrich) was added to each slide, covered with a coverslip, and analyzed at a 20x magnification using a fluorescence microscope (Olympus BX60, United Kingdom) that had been equipped with excitation (510–550 nm) and barrier (590 nm) filters.

DNA damage was evaluated using visual classification of comets into five levels according to comet tail size from 0 (undamaged with no tail) to 4 (maximally damaged with long tail). The DNA damage extent was expressed in arbitrary units (a.u.). Three hundred randomly selected cells (100 cells from each of three replicate slides) were analyzed from each animal, and three AU values were generated for each animal, which were averaged to obtain the final result per animal. The group damage index (DI) ranged from 0, in which all of the cells were undamaged (300 cells × 0), to 1200, in which all of the cells were maximally damaged (300 cells × 4) [26]. The damage frequency (%) was calculated based on the number of cells with tails versus those without tails [27] and the % DNA damage was the fraction of each damage level relative to all of the comets that were analyzed.

2.7. Intracellular Superoxide Anion Fluorescence Measurement. Nonfluorescent dihydroethidium (DHE) was used for intracellular $\bullet O_2^-$ detection by flow cytometry. Hydroethidine is freely cell permeable and is rapidly oxidized by superoxide to ethidium, which binds to DNA and amplifies the red fluorescence signal. To estimate the $\bullet O_2^-$ content in the cell suspension, 10^5 BM-MNCs were stained with 160 μM DHE, followed by a 30 min incubation at 37°C in the dark to facilitate dye loading. DHE-loaded cells were treated with 10 mM H_2O_2 to oxidize the dye as a positive control. After 5 min of H_2O_2 treatment, the BM-MNCs were washed with PBS and cellular ROS levels were analyzed immediately with a FACSCanto II flow cytometer (Becton Dickinson, San Juan, CA, USA). Ten thousand events were recorded from each sample, and forward and side scatter gates were used to select single cells from clumps and debris. Specific fluorescence intensity was expressed as the median fluorescence intensity from the average of at least three repeated experiments in a.u. Red fluorescence was detected between 564 and 606 nm using a 585/42 bandpass filter. The Data were acquired and analyzed using BD FACSDiva software (BD).

2.8. Statistical Analysis. The data are presented as representative figures or as the means ± SEM. The flow cytometry data are expressed as median fluorescence intensity (MFI) ± coefficient of variation (CV) of 3 repeated and statistically reproducible (Friedman test) measurements of at least five independent animals. Normality was evaluated using the Kolmogorov-Smirnov test. Statistical analysis was performed using Student's *t*-test for comparison of two independent groups, and two-way analysis of variance (Anova) followed by the Bonferroni's *post hoc* test was used for comparison of more than 2 groups. The Mann-Whitney test was used to compare the rank sum for the MFIs from the oxidative stress experiments. P values <0.05 were considered to be statistically significant.

Table 1: Body, ventricular, and kidney weights of 2K1C and Sham mice 14 days after renal artery clipping.

Parameters	Sham (5)	2K1C (5)
Body weight (g)	24 ± 0.5	22 ± 0.8*
Ventricular dry weight (mg)	25 ± 0.7	27 ± 0.7*
Clipped kidney dry weight (mg)	37 ± 1.4	25 ± 4.2*
Contralateral kidney dry weight (mg)	40 ± 2.0	44 ± 1.7
Clipped kidney weight/unclipped kidney weight (mg/mg)	0.94 ± 0.02	0.56 ± 0.09**

Values are the means ± SEM. *$P < 0.05$ and **$P < 0.01$ compared with the Sham animals (student's *t*-test for independent samples).

3. Results

3.1. Body, Heart, and Kidney Weights, Blood Pressure, Heart Rate, and Plasma Ang II. Initial body weight was statistically similar among the groups. At the end of the experiments, body weight was reduced and ventricular weight was increased in the hypertensive group compared with the Sham group (Table 1). The hypertensive group also showed a significant increase in ventricular weight compared with the Sham group. Fourteen days after clip application, the left kidney was atrophic, while the right kidney displayed compensatory hypertrophy in the 2K1C mice.

Figure 1 shows the average values of direct resting MAP and HR measurements in conscious animals 14 days after renal artery clipping. As expected, MAP was 40% higher ($P < 0.01$) in the 2K1C than in the Sham mice. Hypertension in the 2K1C mice was accompanied by tachycardia when compared with the Sham mice. Plasma Ang II concentration was 4.5-fold greater ($P < 0.01$) in the 2K1C than in the Sham mice as measured by HPLC.

3.2. Bone Marrow Mononuclear Cells Analysis. We first assessed the effect of Ang II-dependent hypertension on BM-MNC viability and number using a Neubauer chamber after BM separation with a density gradient. Cell viability was assessed using the trypan blue exclusion method, and no differences were found between the groups (Sham: 97 ± 0.54% versus 2K1C: 96 ± 0.54%). To investigate whether the MNC number was reduced in the 2K1C mice compared with the Sham mice, we quantified lymphocytes and undifferentiated cells. As shown in Figure 2, the 2K1C mice had increased

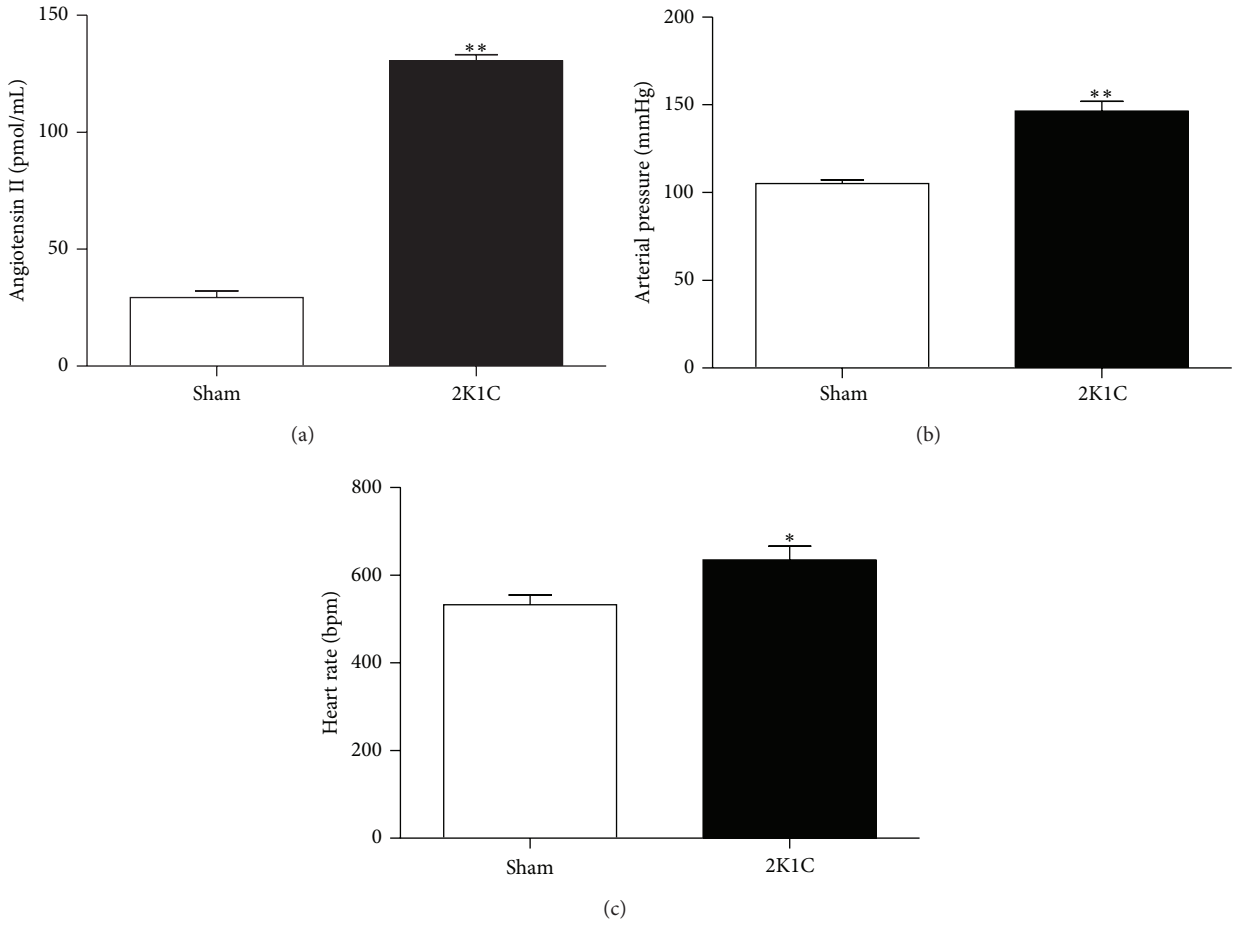


FIGURE 1: Bar graphs demonstrating resting mean arterial pressure, heart rate, and plasma angiotensin II values in conscious Sham ($n = 5$) and renovascular hypertensive (2K1C, $n = 5$) mice. Values are the means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the Sham group (Student's t test for independent samples).

lymphocyte counts (62%) with a simultaneous reduction in undifferentiated cell number (18%) compared with the control animals.

3.3. DNA Damage Measurement with the Comet Assay. The comet assay is a versatile and sensitive method for quantifying and analyzing DNA fragmentation in individual cells that can be used to assess oxidative DNA damage. The basic principle of the comet assay is DNA electrophoresis in an agarose matrix. Because the fragmented DNA migrates, the cells look like a comet under microscope with a head containing intact DNA and a tail containing DNA fragments [24]. Genomic DNA fragmentation incidence was visually analyzed according to comet appearance. To elucidate Ang II-dependent hypertension effects on BM-MNC, DNA damage was scored into five classes according to tail size and the relative tail DNA content indicates the amount of DNA damage. A significant predominance of low genotoxicity levels 0 and 1 in the Sham animals (level 0: 28 ± 3.4 and level 1: 40 ± 2.4%) compared with the 2K1C mice (level 0: 7±4.4 and level 1: 7±1%) was observed. In contrast, severe genotoxicity levels 3 and 4 prevailed in the 2K1C mice (level 3: 36 ± 3.2 and level 4: 27 ± 6%) compared with the Sham mice (level 3: 7 ± 0.4% and level 4: 2 ± 0.4%). Genotoxicity levels are demonstrated as typical images and average values in Figure 3. Moreover, DNA fragmentation was quantified using the DNA damage index and frequency (Figures 3(a) and 3(b)). The 2K1C mice had increased DNA damage as indicated by a higher damage index (Sham: 345 ± 19 versus 2K1C: 806 ± 55 a.u.) and frequency (Sham: 72 ± 3% versus 2K1C: 93 ± 4%).

3.4. Ang II-Dependent Hypertension Induced ROS Production. The above findings led us to further investigate the effects of Ang II-dependent hypertension on ROS production in BM-MNC. DHE is a membrane-permeable blue fluorescent dye that rapidly accumulates in the cytoplasm, where it is oxidized by $\bullet O_2^-$, resulting in red nuclear fluorescence that can be measured by flow cytometry. In Figure 4, representative histograms (Figure 4(a)) and average (bar graphs) $\bullet O_2^-$ production values are shown. As demonstrated by the right shift and in the bar graph, DHE median fluorescence intensity (MFI) values were significantly higher in the 2K1C than in the Sham group (16856 ± 5809 versus 2051 ± 336 a.u., $P < 0.01$), indicating increased intracellular BM-MNC oxidative stress.

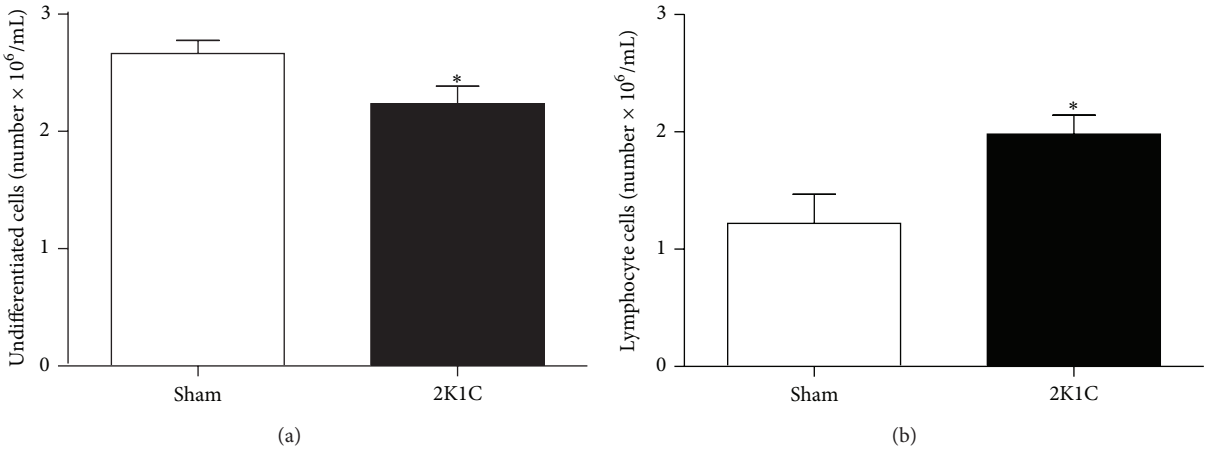


Figure 2: Neubauer chamber analysis of bone marrow mononuclear cells from Angiotensin-II-dependent hypertensive (2K1C, $n = 10$) and normotensive (Sham, $n = 10$) mice. Values of lymphocytes and undifferentiated cells are the means ± SEM. $^{*}P < 0.05$ versus Sham group (Student's t test for independent samples).

4. Discussion

The main finding of this study was marked DNA fragmentation in BM-MNC from Ang II-dependent hypertensive mice, most likely because of augmented $\bullet O_2^-$ production and consequent oxidative stress. Further studies including other experimental models should be designed to discriminate the relative influence of hypertension and Ang II on this process.

Renal artery clipping is accompanied by activation of the RAS and hemodynamic alterations [14, 28–30]. Higher levels of plasma renin and Ang II in 2K1C mice have been observed between 7 and 14 days after clipping and have returned to normal values by day 28 [14, 28, 30–32]. Based on these observations and on a previous publication from our laboratory [2], we performed this study two weeks after renal artery clipping. As expected, 2K1C mice showed atrophy of the clipped kidney and hypertrophy of the contralateral kidney. 2K1C mice exhibited high blood pressure levels accompanied by tachycardia, in agreement with previous studies [2, 5, 24]. In this study, we confirmed the high plasma Ang II, corroborating the concept that RAS activation plays a pivotal role in hypertension development in this murine model. In addition to the pressor and positive chronotropic effects, Ang II also stimulates cardiomyocyte protein synthesis [33–36], which in addition to hypertension may explain the cardiac hypertrophy that we observed in the 2K1C hypertensive mice. On the other hand, future studies should consider the measurement of protein levels of the ventricles as an index of hypertrophy. Taken together, these data suggest that the 2K1C mouse exhibits the main features of endogenous Ang II-dependent hypertension at this time point.

In addition to the systemic actions of the RAS, many tissues and organs have a local RAS, which can have paracrine, autocrine, and intracrine functions [37]. BM is the major reservoir for adult organ-specific stem cells, including endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs), and mesenchymal stem cells (MSCs). In this context, the presence of a complete local BM RAS that affects physiological and pathological blood cell production was hypothesized by Haznedaroglu et al. [38] and has recently been confirmed [9]. In our study, we found augmented lymphocytes and diminished numbers of undifferentiated BM cells in 2K1C hypertensive mice. Considering the presence of RAS components in HSCs [39] and stromal/MSCs [9], it is reasonable to propose that RAS may also be locally activated in BM of Ang II-dependent hypertensive mice. However, this possibility still needs to be confirmed by subsequent studies.

Accumulating evidence suggests that the local RAS is actively involved in BM cells proliferation, differentiation, and death. Of note, Ang II affects the entire BM-MNC pool, such as EPCs [40], HSCs [41, 42], and MSCs [9]. As recently demonstrated, Ang II consistently decreases the number of cultured EPCs through activation of AT1 receptors and induction of apoptosis [40]. In addition and considering that 2K1C hypertensive mice exhibit endothelial dysfunction [23], Ang II could activate inflammatory cells or cytokine production, which may be responsible for cell recruitment in inflammation [43–47]. Moreover, this vasoactive peptide directly stimulates erythropoiesis by augmenting erythropoietin hormone production [48], which regulates erythrocyte differentiation [49], through AT1 receptors [8, 41]. Accordingly, da Cunha et al. [50] and Cassis et al. [48] reported that angiotensin converting enzyme (ACE) inhibitors and AT1 receptor antagonist treatments cause anemia, demonstrating hematopoietic side effects of RAS blockers and indicating that Ang II plays an important role in hematopoiesis. Taken together, experimental evidence suggests that Ang II exhibits important hematopoietic effects by stimulating erythroid, myeloid, and lymphoid differentiation, resulting in augmented lymphocyte number and simultaneously diminished undifferentiated cell number.

There is growing evidence that increased oxidative stress, which results in excessive ROS generation, plays a role in cardiovascular diseases including hypertension, as recently reviewed by us and others [24, 51–53]. Because there is a link between ROS and RAS signaling [53–55], a key mechanism

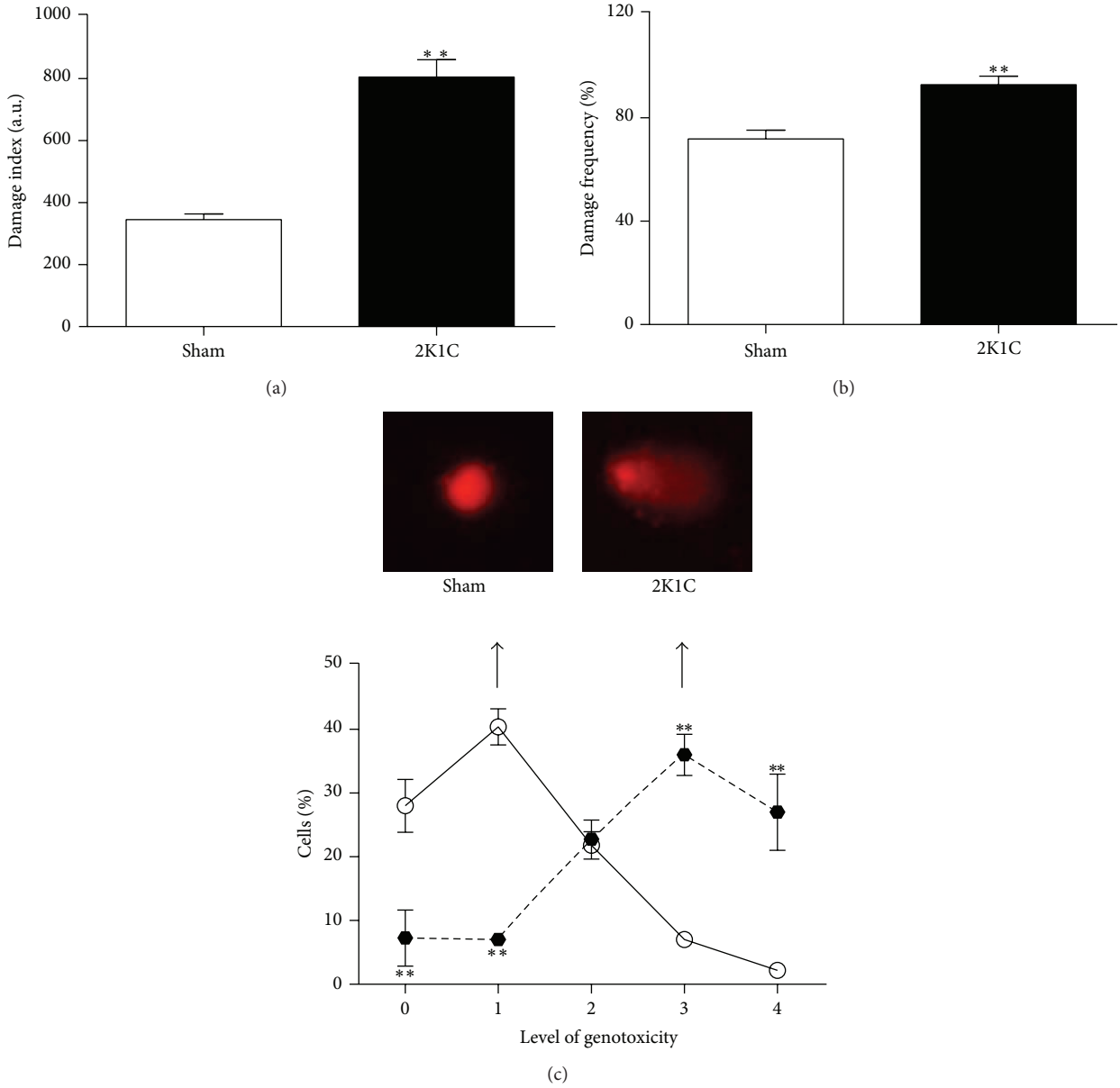
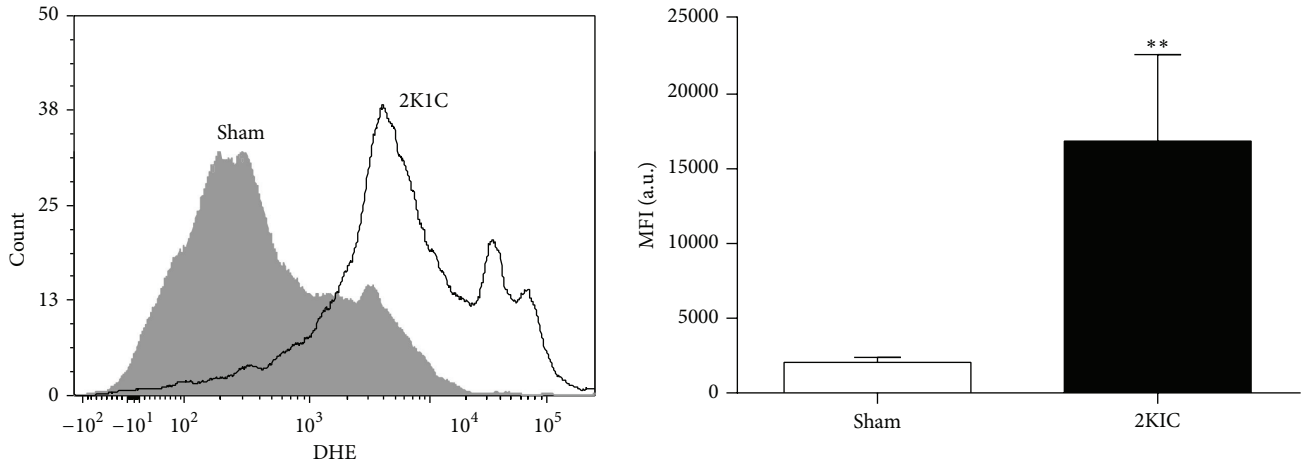


Figure 3: Bone marrow mononuclear cell DNA damage assessed by the comet assay. The left bar graphs demonstrate the average DNA damage index (a) and frequency (b). $^{**}P < 0.01$ versus the Sham group (Student's t test for independent samples). Fluorescent images (c) are typical comets demonstrating increased DNA fragmentation in a renovascular hypertensive (2K1C) mouse compared with a normotensive (Sham) mouse. The lines graph (c) demonstrates the average percentages of DNA damage percentages for each genotoxicity level, comparing the 2K1C (filled circles, $n = 5$) with the Sham (empty circles, $n = 6$) mice. $^{**}P < 0.01$ versus the Sham group (two-way Anova). Values are the means ± SEM.

by which Ang II influences heart and vessel function could be via its ability to activate ROS production [24, 56, 57]. We observed pronounced DHE MFI augmentation in BM-MNC in the 2K1C hypertensive mice compared with Sham normotensive mice. The relationship between oxidative stress and increased blood pressure has been reported in many hypertensive animal models, including the SHR [58], DOCA-salt [59], the 2K1C [14], and the 1K1C [60], which have excessive $\bullet O_2^-$ production due to augmented NAD(P)H oxidase activity [61–64]. Interestingly, in the p47phox knockout mouse with concurrent 2K1C hypertension augmented ROS production occurs via expression of this NAD(P)H oxidase subunit [14]. This enzyme can be activated by hemodynamic forces and vasoactive agonists, for example, Ang II [65–67], which is a powerful vasoconstrictor involved in hypertension pathogenesis that uses ROS as an intracellular signaling mediator [66]. In addition, it seems that Ang II induces the increase of ROS production in EPCs and that this oxidative stress accounts for the Ang II-mediated reduction of EPC number, as this effect can be blocked by cotreatment



(a) (b)

Figure 4: Effects of renovascular hypertension on oxidative stress in bone-marrow-mononuclear cells (BM-MNCs). (a) contains representative histograms of intracellular oxidation of dihydroethidium (DHE) to ethidium by BM-MNC from a hypertensive (2K1C) and a normotensive (Sham) mouse as evaluated by flow cytometry. The bar graph summarizes the median fluorescence intensity (MFI) values of DHE-loaded BM-MNC from 2K1C ($n = 5$) and Sham ($n = 5$) mice. Values are the medians ± coefficient of variation. $^{**}P < 0.01$ versus the Sham group (Mann-Whitney test).

with an antioxidant [40] and it increases gp91phox expression in EPCs, which may contribute to oxidative stress [68].

As discussed above, the role of Ang II goes beyond controlling circulatory homeostasis as discussed above in the impact of this peptide in ROS production, which is stimulated by NAD(P)H oxidase activation [69]. Recent experimental studies have shown that, at high concentrations, ROSs such as $\bullet O_2^-$ are capable of direct protein and lipid oxidation, which causes DNA fragmentation [70]. DNA damage, which frequently occurs in cells exposed to oxidative stress [71], is a form of cellular injury that contributes significantly to the development and progression of cardiovascular disorders [24, 64, 72].

The comet assay has been used to determine DNA fragmentation in blood cells in murine models of spontaneous atherosclerosis [73, 74] and renovascular hypertension [24]. However, this is the first time that the comet assay has been used to assess DNA fragmentation in BM-MNC from 2K1C mice. Our results clearly demonstrated augmented DNA fragmentation in BM-MNC from the 2K1C mice compared with the Sham mice. Augmented DNA damage has also been demonstrated in other animal models of hypertension, including kidney cells from DOCA-salt rats [75] and mouse infused with Ang II [21]. Furthermore, DNA damage caused by ROS occurs more commonly in hypertensive than in normotensive patients and can be reduced by antioxidant drugs [76]. Of note, in the perfused mouse kidney, DNA damage was caused by Ang II, not by induced vasoconstriction, since another vasoconstrictor did not cause DNA damage [21]. Moreover, Ang II induces genomic damage in cultured kidney cells most likely via oxidative mechanisms, which can be prevented by AT1 receptor antagonists and by antioxidants [77]. The 2K1C mice had increased $\bullet O_2^-$ production, which is a highly reactive and short-lived radical that is responsible for ROS generation and can interact with nearby molecules, such as DNA [21, 78, 79]; thus, our data suggest that ROS plays a key role by inducing DNA oxidative damage in this model of Ang II-dependent hypertension.

In conclusion, we demonstrated that arterial hypertension induced by endogenous RAS activation by clipping the renal artery for two weeks (the 2K1C mouse model) results in a marked increase in ROS production with consequent BM-MNC DNA damage. We speculate that Ang II effects may be due to circulating and local BM RAS; therefore, both systems may play a crucial pathobiological role in the DNA damage observed in BM-MNC of 2K1C hypertensive mice. Taking into account that BM-derived cells are responsible of maintaining, generating, and replacing differentiated cells as a consequence of physiological cell turnover or tissue damage due to injury, the data obtained by this study suggested that comorbidities, specifically Ang II-dependent hypertension, have to be particularly considered if autologous transplantation is intended, since the donor tissue (i.e., bone marrow) might be altered in its functionality.

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