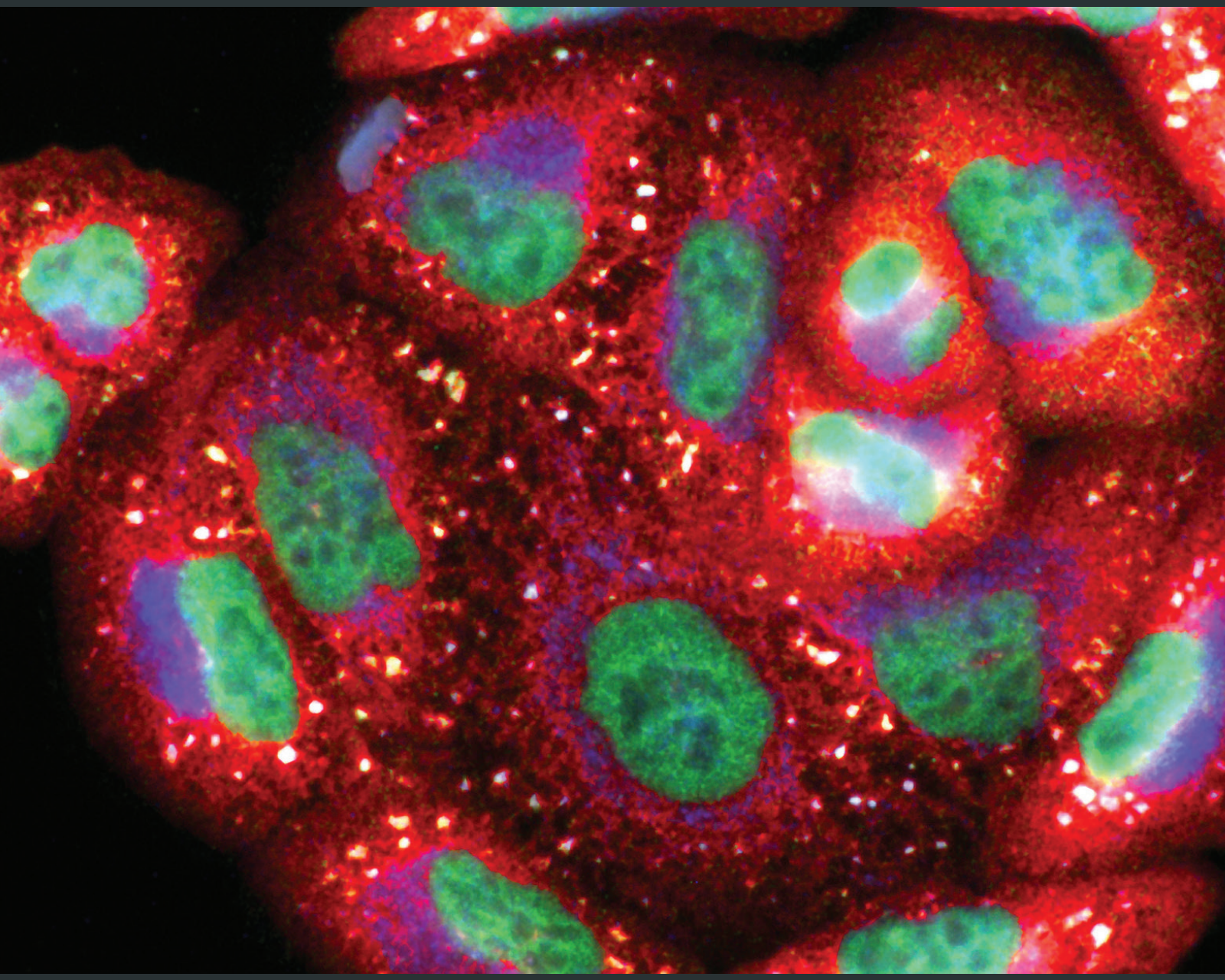
Oxidative Medicine and Cellular Longevity

New Insights into the Role of Oxidative Stress in Onset of Cardiovascular Disease

Lead Guest Editor: Adrian Doroszko
Guest Editors: Aneta Radziwon-Balicka, Robert P. Skomro, and Piotr Dobrowolski



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Editorial

New Insights into the Role of Oxidative Stress in Onset of Cardiovascular Disease

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Cardiovascular disease is one of the major healthcare problems of the world population. Understanding its determinants is essential for designing effective therapeutic interventions. Cardiorespiratory failure is still among the most common reasons of mortality and morbidity. Since advances in molecular medicine have enabled us to identify the crucial regulatory mechanisms of pathophysiological pathways, it is now possible to develop novel therapeutic strategies, based on evidence from molecular studies.

Numerous studies have shown that endothelium plays a pivotal role in the maintenance of appropriate vascular tone and structure and all the disturbances initiating the onset and promoting the progression of atherosclerosis come from these cells. Vascular endothelium is critically located between blood and the vascular wall and is among the largest endocrine organ in terms of surface (400–4000 m^2), its mass reaching 1.5 kg. Under physiological condition, by releasing numerous mediators of auto- and paracrine action, it performs the anti-inflammatory, antiaggregatory, vasodilative, and antiproliferative functions.

All cells are capable of producing reactive oxygen species (ROS), and some evidence suggests that ROS produced by cardiac myocytes and vascular smooth muscle cells play an important role in the development and progression of cardiovascular disease. Endothelial dysfunction is characterized by decreased vasodilative potential as well as increased inflammatory and aggregatory activity. The mechanisms underlying decreased vasodilative action include decreased nitric oxide (NO) bioavailability, oxidative stress, changes in the arachidonic acid metabolite biotransformation, and activation of the renin-angiotensin-aldosterone system (RAAS). All the pathological mechanisms lead to increased platelet aggregation, inflammatory reaction in the vascular wall promoting development of atherosclerosis and its consequences. Endothelial activation under pathological condition is associated with increased mortality in numerous diseases.

Since the platelets are among the most important determinants of pathological modifications of vascular repair, the use of functional proteomics should enable the assessment of the factors affecting an individual platelet functional variability. In addition, a paracrine effect of endothelium on platelet function is an important issue. It is well known that activation of endothelium activates platelets and that antiplatelet drugs improve endothelial function—for instance, platelet aggregation may be inhibited by nitric oxide (NO). The platelet NO concentration is low and it seems that no expression of the nitric oxide synthase in platelets is present. However, the effect of NO on platelets is mediated through the activation of the platelet guanylyl cyclase and production of cGMP.

This special issue is aimed at stimulating the continuing effort to understand the molecular mechanisms of cardiovascular damage induced or mediated by oxidative stress.

M. Jakubowski et al. in a proteomic LC/MS study analysed the molecular mechanism underlying aspirin resistance. Interestingly, the authors found that carbonic anhydrase II, a forgotten enzyme, described for the first time over 30 years ago, was the only discriminatory protein affecting aspirin responsiveness. Increased activity and/or concentration of CA II in platelets should be rated as a new independent risk factor for aspirin resistance and thus for thromboembolic events. Since numerous carbonic anhydrase inhibitors are already well known, and registered for use in humans, the authors postulate to use these drugs in clinical setting, especially in patients with increased platelet activity/amount of carbonic anhydrase. Among the CA II-dependent mechanisms modifying platelet responsiveness, the pH changes of platelet cytosol leading to impaired acetylating of cyclooxygenase by ASA are noteworthy. This in turn could affect the antiplatelet effect of ASA as well as platelet inflammatory activity and energetic metabolism. The CA II activity in platelets has been hypothesized since the proton efflux following platelet thrombin stimulation was described. This was followed by demonstration of the presence of CO_2 hydration in platelets, which was inhibited by ethoxzolamide, a carbonic anhydrase II inhibitor. Interestingly, carbonic anhydrase II was found to catalyze generation of nitric oxide form nitrite, which may be an additional origination of intraplatelet NO. This reaction is significantly enhanced by both decreased pH and dorzolamide, which are inhibitors of CAII main activity.

Peroxynitrite ($ONOO^-$) is a highly reactive oxidant which is generated from the coupling between the endothelial nitric oxide and superoxide. Its detrimental action on the development of cardiac injury as well as its negative effects on cardiac systolic function has been well established. P. Rola et al. intended to investigate if nitric oxide, a potent antiplatelet factor, could be a potential transmitter of the low-level laser therapy-induced modification of platelet activity. In order to explore the impact of the low-level laser therapy on platelet activation, the levels of the platelet factor 4 (PF-4) and sP-selectin were measured both at baseline and following the laser irradiation. The authors have demonstrated that the low level laser therapy decreases the whole-blood platelet aggregation regardless of the NO bioavailability or changes in the platelet activation markers.

J. Sun and colleagues in a review paper indicate that endothelial dysfunction and endothelial nitric oxide synthase (eNOS) dramatically increase the rate of abdominal aortic aneurism (AAA) formation in animal models, pointing at the involvement of endothelium in AAA pathogenesis as well as at possible pharmacological endothelium-related targets in limiting the onset of this disorder. Some authors postulate that AAA and atherosclerosis constitute two separate but related diseases, based on their different clinical patterns. Since there is a paradoxical differential correlation between diabetes and aneurysm formation in the retinal capillaries and the aorta, the authors postulate that deciphering the significance of such a difference could provide better therapeutic strategies for AAA management.

K. Kikuchi et al. examined the effect of edaravone (a reactive oxygen species scavenger) on the thrombolytic effectiveness of alteplase by measuring thrombolysis using a microchip-based flow chamber assay. The thrombolytic effect of alteplase was significantly attenuated in the presence of hydrogen peroxide, suggesting that oxidative stress may limit the thrombolysis. Edaravone alone did not influence thrombosis nor thrombolysis but enhanced alteplase-mediated thrombolysis in vitro, likely by acting as an antioxidant to prevent free radical-related inhibition of alteplase activity on thrombi. Furthermore, edaravone significantly attenuated inhibition of alteplase-induced fibrinolysis by hydrogen peroxide. It would be very interesting to analyse these findings with respect to the risk for severe bleedings related to thrombolytic therapy.

In a review paper, J. Zhang and colleagues discuss how temporal dynamics and localization of activated AMPK and PKA enzymes play a pathogenic role, that is, in diabetes, and propose therapeutic strategies aiming at localized PKA and AMPK signalling to reverse mitochondrial dysfunction, oxidative stress, and death of cardiac and endothelial cells during ischemia and diabetes.

On the other hand, X. Gu et al. reported that acetaldehyde dehydrogenase 2 (ALDH2) is expressed in cardiac fibroblasts and that high glucose concentration may increase oxidative stress-mediated reaction, decrease ALDH2 activity and expression, and induce cardiac fibroblast apoptosis and fibrosis. Activation of ALDH2 may be a defence mechanism by ameliorating the high glucose-induced cardiac fibroblast fibrosis by decreasing oxidative stress and apoptosis.

A. Stanek et al. in a research study demonstrate that increased oxidative stress as well as higher serum concentrations of placental growth factor—PlGF and sCD40L—and increased intima media thickness in carotid arteries may reflect the acceleration of atherosclerosis in male patients in the active phase of ankylosing spondylitis and without concomitant classical cardiovascular risk factors. The authors observed the increased concentration of lipid peroxidation products (malonyldialdehyde (MDA)) in plasma and erythrocytes which could play an important role in LDL modification and their deviation towards macrophages.

To summarize, the manuscripts published in this special issue present recent developments in assessment of cardiovascular consequences of oxidative stress including modulation in platelet aggregation, coagulation, or fibrinolysis. Importantly, these papers also unmask many challenging issues and present novel therapeutic approaches. All articles involved in this special issue had brought about new and valuable information on the role of oxidative stress in the development of cardiovascular disease. We believe that some of the presented studies will provide new evidence, which could lead to the discovery of potential drug targets for the development of new therapeutic approaches for combating cardiovascular disease in the future.

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Review Article

Endothelium as a Potential Target for Treatment of Abdominal Aortic Aneurysm

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Abdominal aortic aneurysm (AAA) was previously ascribed to weaken defective medial arterial/adventitial layers, for example, smooth muscle/fibroblast cells. Therefore, besides surgical repair, medications targeting the medial layer to strengthen the aortic wall are the most feasible treatment strategy for AAA. However, so far, it is unclear whether such drugs have any beneficial effect on AAA prognosis, rate of aneurysm growth, rupture, or survival. Notably, clinical studies have shown that AAA is highly associated with endothelial dysfunction in the aged population. Additionally, animal models of endothelial dysfunction and endothelial nitric oxide synthase (eNOS) uncoupling had a very high rate of AAA formation, indicating there is crucial involvement of the endothelium and a possible pharmacological solution targeting the endothelium in AAA treatment. Endothelial cells have been found to trigger vascular wall remodeling by releasing proteases, or recruiting macrophages along with other neutrophils, into the medial layer. Moreover, inflammation and oxidative stress of the arterial wall were induced by endothelial dysfunction. Interestingly, there is a paradoxical differential correlation between diabetes and aneurysm formation in retinal capillaries and the aorta. Deciphering the significance of such a difference may explain current unsuccessful AAA medications and offer a solution to this treatment challenge. It is now believed that AAA and atherosclerosis are two separate but related diseases, based on their different clinical patterns which have further complicated the puzzle. Therefore, a thorough investigation of the interaction between endothelium and medial/adventitial layer may provide us a better understanding and new perspective on AAA formation, especially after taking into account the importance of endothelium in the development of AAA. Moreover, a novel medication strategy replacing the currently used, but suboptimal treatments for AAA, could be informed with this analysis.

1. Introduction

By definition, an aneurysm is the dilation of an artery to a diameter at least 50% greater than its normal size. In general, aortic aneurysms are anatomically divided into thoracic aortic aneurysm (TAA) and abdominal aortic aneurysm (AAA) including suprarenal and infrarenal aortic aneurysms. The incidence of TAA each year ranged from 6 per 100 000 in a British study [1] to 9.1/16.3 (F/M) per 100 000 in Sweden and 17.39/21.75 (F/M) in Spain [2, 3]. It is estimated that three quarters of all aneurysms occur in the abdominal aorta. The annual incidence of abdominal aortic aneurysm is 40 for every 100 000 population [4]. Six times more men than women are affected [4, 5]. In elderly males, general infrarenal abdominal aortic diameter is about 15–24 mm. If the diameter exceeds 30 mm, usually more than two standard deviations above the mean diameter for both men and women, this condition is conventionally regarded as AAA [6–8]. A UK survey showed 1.5% of the population had an AAA measuring >30 mm [9]. The Multicenter Aneurysm Screening Study in the USA reported that 4.6% of the population between the age of 65 and 74 years had an AAA [10]. The prevalence of men over 65 years with AAA in Sweden was 1.8% [11]. A meta-analysis of a collection of international studies showed that AAA in men and women is about 6% and 1.6%, respectively [12]. It is suggested that AAA is more

common in the clinic than TAA. In recent years, incidence of AAA is trending upward with the aging of the population. Moreover, compared to TAA, AAA has a higher morbidity and poses a greater danger to the population because of its faster rate of growth [8].

Aneurysm rupture is the most serious consequence of AAA. Often the presence of AAA is unknown until rupture, which rapidly progresses to exsanguination and death. The total mortality rate for AAA rupture reaches 90%; therefore, the ideal management is to repair the aneurysm before rupture occurs. However, many aneurysms do not reach surgical indications when they are first discovered, but their size will increase year by year. Blood vessel diameter is strongly correlated with higher rupture risk. It is believed that AAA over 6 cm has a 26% annual risk of rupture [13]. Consequently, AAA has been referred to as "a silently ticking time bomb" in the body [14].

2. Anatomy of Abdominal Aorta Is Different from the Thoracic Aorta

Thoracic and abdominal aortas have structural differences in the number of lamellar units, which range from 55–60 U and 28–32 U, respectively [15]. Cells from different segments of aorta have a clear difference in their origin including from the neural crest, mesenchyme, and splanchnic mesoderm, which correspond to different segments. Moreover, the neural crest cell precursors of the thoracic aorta respond differently to various cytokines and growth factors than the mesodermal precursors of the abdominal aorta [16]. Regional differences between the thoracic and abdominal aortas lead to different cellular responses to the same stimuli. For example, the abdominal aorta is susceptible to atherogenesis in contrast to its thoracic counterpart [17]. Moreover, it has been demonstrated that mutations in the transforming growth factor-β (TGF-β) receptor may lead to TAA but have little effect on the abdominal aorta [18]. This may be explained by the differential function of inflammatory pathways or TGF-β between vascular sites. Notably, the medial layer in abdominal aortas normally is completely avascular, whereas the medial layer in thoracic aorta contains vasa vasorum [15]. Therefore, the medial layer of abdominal aorta is more prone to hypoxia than thoracic aorta. Hypoxia is observed in intima and media of AAA lesion because intraluminal thrombus in vascular lesions may prevent luminal perfusion of oxygen, contributing to hypoxia in intima [19]. Furthermore, arteriosclerosis and intimal hyperplasia were shown to induce stenosis of the adventitial vasa vasorum that aggravates tissue hypoxia in the abdominal aorta [20].

3. Pathological Mechanisms of AAA

Although abdominal aortic aneurysmal dilatation is caused by various etiologies leading to complex pathogenesis, histopathological results are always similar; for example, there are degenerations mainly in media and adventitia of aortic wall. Classically, occurrence of aortic aneurysm is associated with aortic wall defects and damage consequent to inflammation, oxidative stress, matrix metalloproteinases (MMPs) activation, and apoptosis of vascular smooth muscle cells (VSMCs). Specifically, inflammatory cells infiltrate in the media and adventitia due to autoimmune reaction with extrinsic antigens, inducing oxidative stress and overproduction of cytokines/chemokines and proteases. This process leads to the breakdown of elastic fibers, degradation of collagen fibers, and loss of VSMCs (Figure 1). As a result, the aortic wall is weakened because of decreased thickness and reduced mechanical function. Eventually, the aortic wall cannot tolerate the impact of blood flow and dilates to form AAA.

4. Genetics and AAA

Abdominal aortic dilatation is the outcome of both environmental and genetic factors. In minors, thoracic aortic aneurysm in particular is always the direct consequence of mutations in key genes such as *FBN1*, *COL3A1*, *ACTA2*, and *TGFBR1/2* that causes vassal wall structural defects [21]. However, previous studies have proposed that AAA is a multifactorial disease which involves many genes and pathways, such as extracellular matrix (ECM), inflammation, immunity, oxidative stress, cell signaling, cell growth, and cell survival. Among them, genetics still poses a pivotal role in the AAA development and formation since the risks are higher in those patients with AAA family history than without [22]. Recently, exome sequencing has been carried out to explore possible candidate gene variants by comparing DNA tissue samples from AAA patients and normal controls, in which mutations in 25 genes were found to be associated with AAA formation. However, association studies cannot validate the causative factors for AAA which are still poorly understood [22]. Among them, a missense mutation in ESRRA (estrogen-related receptor alpha), a vascular endothelial growth factor (VEGF) regulator, has been identified [23]. Therefore, there is genetic evidence for endothelium involvement in AAA formation.

5. Atherosclerosis and AAA

The infrarenal abdominal aorta is related with the atherosclerotic development and is coincidently the most common site of abdominal aneurysm formation. Previously, it was believed that atherosclerosis is the main pathological process leading to AAA formation. This belief was based on the findings that patients with AAA frequently have concurrent atherosclerosis [24]. However, it has been suggested that aneurysm formation and atherosclerosis do not develop in parallel, but through different pathogenic mechanisms independently [24]. For example, the lumen narrows in atherosclerosis but grows larger in AAA; diabetes precipitates atherosclerosis but protects against AAA; and proliferation of SMC happens in atherosclerosis but apoptosis of SMC in AAA. Therefore, AAA and atherosclerosis are now regarded as two separate but related diseases. Indeed, atherosclerosis and AAA share some common risk factors such as both are closely associated with endothelial dysfunction [24].

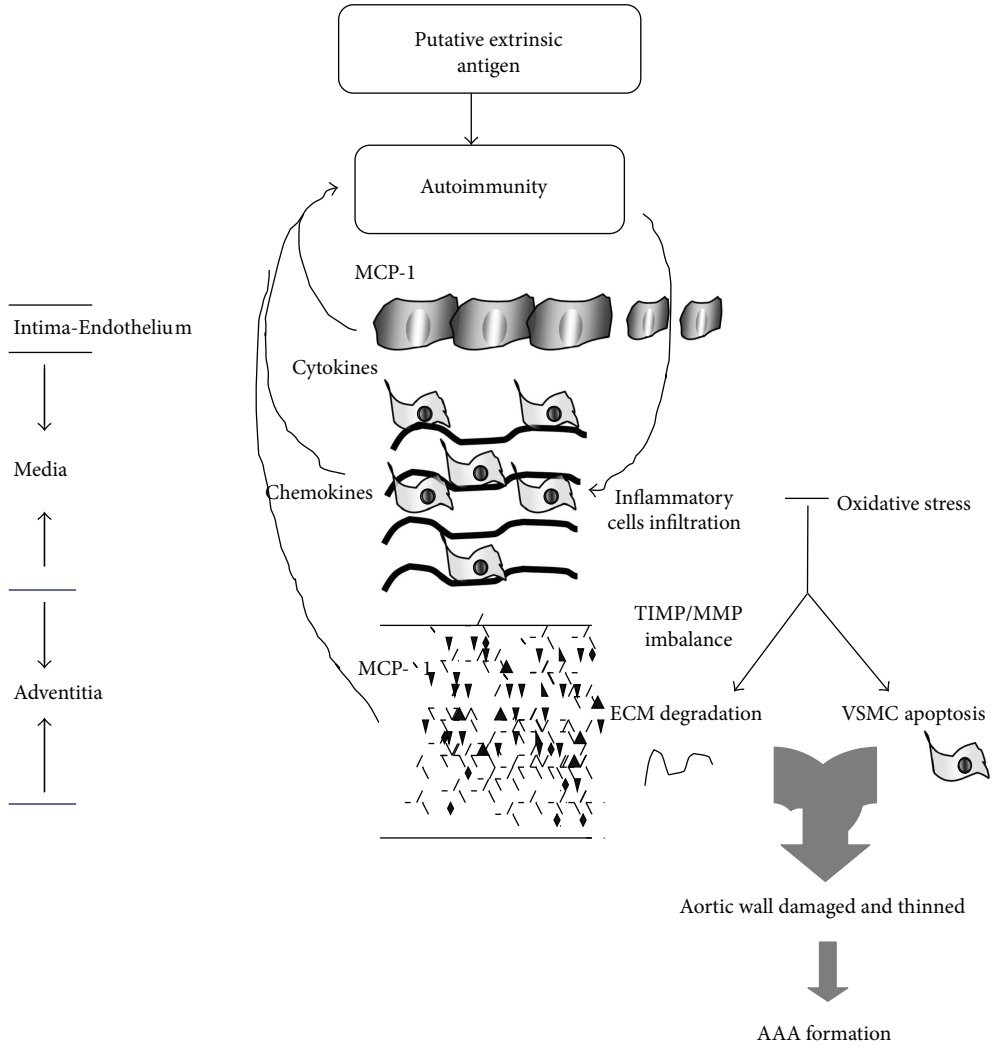

Figure 1: The classic putative mechanism for AAA formation. Extrinsic antigens such as protein from microorganisms cause autoimmunity which can cross-react the medial layer of the aortic wall. This induces and amplifies the autoimmune reaction through inflammatory cell infiltration, cytokine and chemokine production in the three layers of the vascular wall, oxidative stress, and so on. All of these responses further damage the vascular wall via induction of SMC apoptosis and ECM degradation due to TIMP/MMP disorder. VSMCs: vascular smooth muscle cells; ECM: extracellular matrix; TIMP: tissue inhibitors of metalloproteinases; MMP: matrix metalloproteinase; MCP-1: monocyte chemoattractant protein-1.

6. Animal Evidence for Endothelial Dysfunction in AAA

It is generally believed that the receptor inducing cell response to increase tension in the aortic wall produced by Ang II originates in the medial layer but not in the endothelium. Conversely, endothelial cell-specific deficiency of Ang II type 1a receptors markedly attenuates the development of Ang II-induced aortic aneurysms in LDL receptor$^{-/-}$ mice, but Ang II type 1a receptor deficiency in VSMCs had no such effect on the development of aneurysms [25]. Moreover, Franck et al. demonstrated that restoring the endothelial lining is an efficient therapy to control AAA dynamics and stop AAA expansion [26].

Gradually, the endothelium drew the attention of AAA research. Endothelial cells are single layer of squamous epithelium lining the vessel lumen and participate in many physiological activities to maintain the normal structure and function of the vessel wall. In the arterial wall, endothelial cells secrete a variety of substances that influence the function of other cells, including smooth muscle cells, white blood cells, and so on, to initiate a critical series attacks on media and adventitial layers. The pathological changes of endothelial cells or endothelial dysfunction are possible earlier than those of media and adventitia in the process of AAA formation.

Deficiency of eNOS increases atherosclerosis and AAA in *apoE* (−/−) mice without altering arterial blood pressure, body weight, serum cholesterol concentrations, or distribution of lipoprotein cholesterol [27, 28]. Moreover, Ang II-infused apoE (−/−) or PCSK9 overexpression mice is induced by hypercholesterolemia and AAA [29]. The endothelial function is regulated by NO bioavailability whose production is modulated by eNOS expression and coupling status.

However, eNOS uncoupling leads to endothelial dysfunction, promoting excessive oxidative stress in endothelial cells which induces harmful oxidative/nitrosative stress to local cells.

The coupling status of eNOS is determined by its cofactor tetrahydrobiopterin (HB_4), whose production is governed by a de novo synthetic pathway (the key enzyme being GTP cyclohydroxylase 1 (GCH1)) and a salvage pathway (the key enzyme being dihydrofolate reductase (DHFR)). It has been reported that the mouse model (hph1) with HB_4 deficiency due to GCH1 mutation was characterized as having eNOS uncoupling and endothelial dysfunction, which led to a high risk of AAA with Ang II infusion [30]. Recoupling eNOS with HB_4 supplementation or folic acid treatment prevented AAA formation or reduced such risk [30]. Moreover, H4B levels in serum and the aorta are closely correlated in which both decreased with AAA development but increased with folic acid treatment in Ang II-infused hph1 or apoE (−/−) mice, suggesting serum H4B can be used as a biomarker for AAA development and treatment assessment [31]. Furthermore, Cai et al. reported that animals possessing the double mutation of hph1 and NOX1, NOX2, p47phox, or NOX4 had reduced AAA incidence and abdominal aortic expansion after Ang II infusion due to reduced superoxide production, as well as improved NO and H4B bioavailability, and restored eNOS coupling activity compared to hph1 mice. These changes preserved DHFR function in the endothelium [32].

7. Clinical Evidence for Endothelium Involvement in AAA

A clinical study demonstrated that circulating biomarkers of endothelial dysfunction in atherosclerosis patients (white blood cell count, fibrinogen, D-dimer, troponin T, N-terminal pro-brain natriuretic peptide, and high-sensitivity C-reactive protein) are also closely related to the occurrence and incidence of AAA [33]. Epidemiological surveys show that AAA risk factors, such as aging, smoking, male sex, high blood pressure, hyperlipidemia, atherosclerosis, and hyperhomocysteinemia, are the main factors that cause damage to endothelial cells leading to endothelial dysfunction [34–36]. For instance, the prevalence of AAA in the population who smoke is four times greater than those who do not smoke, meaning smoking is detrimental to endothelial function and thus closely related to many cardiovascular diseases [34, 37]. Furthermore, recent studies in humans indicate that some endothelial protective medications such as statins, angiotensin-converting enzyme inhibitors (ACEIs), and AT_1 receptor blockers (ARBs) may be helpful to inhibit aneurysm rupture and growth, yet the effect is unclear in some larger cohort reports [38, 39]. Mechanism of medication is also not clear, so the effect of regulating endothelial function and increases in nitrogen oxide (NO) cannot be ruled out.

8. Endothelium and Shear Stress

The endothelium is in direct contact with blood flow which can produce a large amount of shear stress. The influence of different flow patterns on vessel walls produces two kinds of shear stress: unidirectional laminar shear stress (LSS) and oscillatory shear stress (OSS). It has been reported that AAA occurs in areas of reflux, low wall shear stress, or blood flow disorders, while high LSS reduces the development of AAA [40]. Hemodynamic forces regulate oxidative stress and reactive oxygen species (ROS) in the endothelium. LSS inhibits superoxide while OSS increases that from endothelial cells. Additionally, NOX mediates oxidative stress in response to distinct shear stresses [41]. LSS activates NOX2 to induce NO production, while OSS activates NOX1 with uncoupled eNOS [41]. Furthermore, shear stress decreases the expression of eNOS in endothelial cells via a receptor named scavenger receptor class B member-1 (SR-B1) [42]. SR-B1, a receptor for high-density lipoprotein, is involved in the regulation of endothelial cells in response to shear stress, which contributes to AAA formation in some patients with atherosclerosis and some animal models such as Ang II-infused ApoE gene knockout mice [28].

Shear stress regulates the expression and activity of proteases secreted by endothelial cells. Normally, ECM is composed of elastin, collagen, proteoglycan, glycoprotein, glycosaminoglycan, and so on. ECM maintains an equilibrium between synthesis and degradation, while patients with AAA have disorders in ECM metabolism, manifesting as lesions of elastin and collagen fibers [43]. MMPs and cysteine proteases, such as cathepsins K, L, and S, participate in the degradation of ECM [44, 45]. Accordingly, expression of TIMP3 decreases and MMPs activity increases in relation to the condition of OSS compared to LSS [46]. Although some studies show that the TIMP is unexpectedly increased in the wall of the aneurysm [47], the balance between proteases and antiproteases seems to favor proteolysis [7]. Cathepsin is another kind of protease family that hydrolyzes the ECM, destroying the elastic and collagen fibers to contribute to the formation of AAA [45]. Expression of cathepsin is observed to be increased in endothelial cells at the site of AAA lesions [48, 49]. The expression and activity of cathepsins are significantly lower under laminar flow than those under turbulent flow in the endothelial cells [50, 51]. Hemodynamic forces indirectly mediate the expression of cathepsin in endothelial cells by regulating infiltration of inflammatory cells and release inflammatory factors [51, 52].

9. Endothelium and Inflammation and Oxidative Stress

Endothelial cells are involved in the inflammation of the aortic wall. Shear stress and blood flow on the endothelium influence the aortic wall via inhibition of inflammatory cytokines and adhesion molecules secreted by endothelium, further reducing the inflammatory reaction in the media and adventitia [53]. Based on previous studies, it is possible that AAA formation is triggered by innate or autoimmunity to extrinsic antigens that may share molecular motifs with those on the aortic wall [54]. Inflammation has been considered to be an essential factor in the initiation and progression of aneurysms. Infiltrating macrophages and leucocytes are major sources of proteinases. Furthermore, infiltrating

immune cells release cytokines, ROS, and cellular adhesion molecules, which lead to further recruitment of immune cells, induction of VSMC apoptosis, and tissue injury.

Evidence shows that transcription factor nuclear factor-kappa B (NF-κB) mediates a number of genes associated with both inflammatory and oxidative reactions in the aortic wall [55–57]. It was found that when the NF-κB pathway of endothelial cells was blocked, infiltration of inflammatory cells, expression of inflammatory factors, and oxidative stress response were reduced in the media and adventitia of arteries; this led to the inhibition of aneurysm formation [58]. Hannawa et al. found that selectins promote the recruitment and infiltration of inflammatory cells in early AAA formation [59]. They further demonstrated that inflammatory cell recruitment was significantly diminished in P-selectin knockout mice, so deficiency of P-selectin, which is expressed in endothelial cells, attenuated aneurysm formation [60].

Endothelial cells are also involved in oxidative stress of the aortic wall. Endothelial cells are stimulated by secreted substances that increase the level of ROS in smooth muscle. The occurrence of oxidative stress in endothelial cells is observed before the changes in VSMCs as well as infiltration of inflammatory cells. Oxidative stress and inflammation interact to increase damage to arterial tissues. At the site of tissues with chronic inflammation, increased level of ROS is generally observed. Inflammatory mediators were shown to activate NADPH oxidase to produce O_2^-, and NADPH oxidase as well as iNOS, in turn, participates in inflammation reaction [61, 62]. ROS products promote the infiltration of inflammatory cells and increase the secretion of proinflammatory cytokines [63]. Besides these roles, ROS directly activates MMPs [64], inhibiting plasminogen activator inhibitor type-1 (PAI-1) which is a MMP inhibitor that induces apoptosis of VSMC [65]. This increase in ROS in endothelial cells altered the function of VSMCs and promoted oxidative stress, in which cyclophilin A (CypA) may act as an intermediate molecule [66]. CypA induces smooth muscle cell migration and proliferation, increases the expression of endothelial adhesion molecules, and mediates the chemotaxis of inflammatory cells [66]. After specific knockdown of the CypA gene in endothelial cells, however, Ang II did not increase the ROS level in VSMCs, in which secretions of inflammatory cytokines such as MCP-1, IL-6, and chemokine were also blocked [67]. Moreover, levels of CypA in VSMCs are also influenced by endothelial cells [66].

10. ECM and VSMCs in Media

ECM and VSMCs are chief components in media tissue. On the one hand, VSMCs decrease in number because of apoptosis and necroptosis [68], and on the another hand, VSMCs synthesize large amounts of ECM components and increase proliferation as well as migration in the vascular wall. This is one of the most significant reasons for media tissue weakening that leads to structural and functional abnormalities in apoptotic VSMCs [69]. Wang et al. reported that macrophage recruitment caused VSMCs death through a FasL/Fas-Caspase-8-RIP1-mediated pathway [70]. RIP1 inhibitors reverse VSMC loss, prevent inflammation and ECM degradation, and promote aortic tissue repair. Additionally, VSMCs are essential not only for short-term regulation of blood pressure via alteration of vessel tone and diameter, but also for long-term adaptation via structural remodeling by changing cell number and connective tissue composition [71]. As contractile properties decrease, proliferation and migration increase in VSMCs of AAA lesions as result of transforming to a synthetic phenotype. It has been demonstrated that inhibition of the mTOR cascade attenuates AAA progression by preserving or restoring VSMC contractile phenotype [72].

Compared to nonruptured aneurysms, angiogenesis is more obvious in the external two thirds of the media tissue in ruptured AAA. The infiltration of inflammatory cells and inflammatory factors causes medial lymphangiogenesis and angiogenesis [19]. These neovessels are incomplete, leaky, and rupture easily, resulting in vascular remodeling and weakening the aortic wall by weakening its structural integrity [73, 74]. Moreover, formation of lymphatic microvessels may introduce inflammatory cells into media tissue and newly formed lymphatic microvessels cannot drain lymph fully [19]. However, the exact mechanism of neovascularization and lymphangiogenesis in aortic aneurysm rupture and development is not clear.

11. Adventitia and AAA

Both the thoracic aorta and the abdominal aorta are elastic arteries that consist of an intimal, medial, and adventitial layer. The alteration of elastin and collagen in the aortic wall is dependent on production of proteases by resident vascular wall cells (medial smooth muscle cells and adventitial fibroblasts) and lymphomonocytic infiltrate cells, a process in which adventitia plays a major role. Notably, adventitial fibroblasts secreting MCP-1 have also been shown to recruit monocytes to the aortic wall that then promote the proliferation of more fibroblasts to amplify the inflammatory response. This amplification represents the pivotal step in AAA development and formation [75].

12. Signaling Pathway and AAA

Many mechanisms have been proposed for AAA formation, and basic research has helped to determine the molecular basis and mediators of aortic damage including angiotensin II, leukotriene-LT4, prostaglandin E2 (PGE2), interleukins, tumor necrosis factor, tissue plasminogen activator, c-Jun N-terminal kinase, NF-κB, rho kinases, osteoprotegerin, chymases, hypoxia-inducible factor-1 (HIF-1), metabolism, SMAD, TGF-β, and its signaling pathway [23, 76, 77]. The overall mechanism is still a puzzle, but the current knowledge about AAA formation is illustrated in Figure 2: in the presence of some aggravating factors such as hypertension, smoking, and aging, NOXs are activated upon RAS activation which further causes eNOS uncoupling via oxidation of H4B (cofactor for eNOS) or oxidative stress/superoxide overproduction. Endothelial dysfunction is consequential to eNOS uncoupling, which triggers a series of cascades including overexpression of ICAM/VCAM to amplify the immune

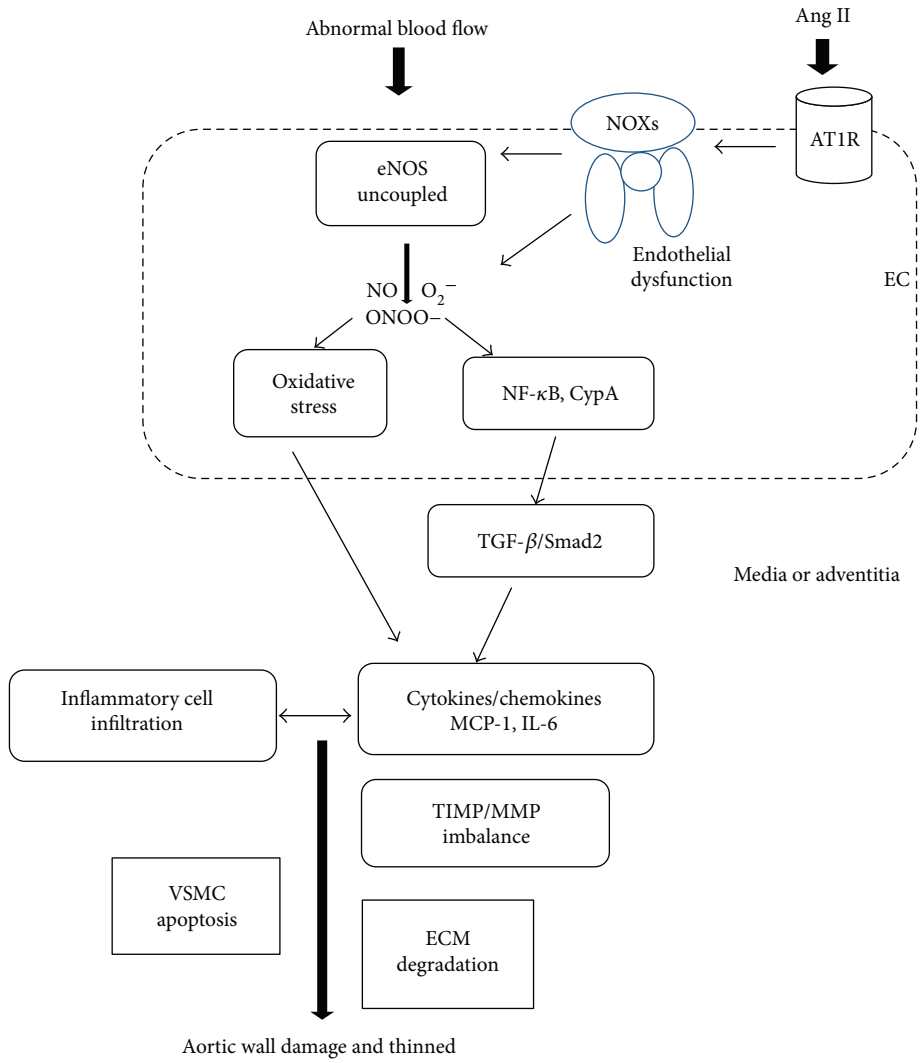

Figure 2: The proposed role of the endothelium in AAA formation. Shear stress produced by blood flow together with Ang II influences the endothelium to cause endothelial dysfunction via uncoupling of eNOS and oxidative stress induction. Endothelial dysfunction activates NOXs, NF-κB, CypA, and TGF-β/Smad2 pathways to further initiate and amplify the inflammatory reaction via overproduction of cytokines and chemokines such as MCP-1 and IL-6. More inflammatory cells infiltrate and exacerbate oxidative stress to form a vicious cycle. The balance of TIMP/MMP is tipped, and proteolysis is promoted. Eventually, the aortic wall is damaged as a result of SMCs apoptosis and ECM degradation due to proteolytic degradation. EC: endothelial cell; Ang II: angiotensin II; AT1R: AT_1 receptor; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; O_2^-: superoxide anion; $ONOO^-$: nitrous oxide ion; NF-κB: nuclear factor-kappa B; CypA: cyclophilin A; TGF-β: transforming growth factor-β; MCP-1: monocyte chemoattractant protein-1; IL-6: interleukin-6; TIMP: tissue inhibitors of metalloproteinases; MMP: matrix metalloproteinase; ECM: extracellular matrix; VSMCs: vascular smooth muscle cells.

response and induce monocyte infiltration. These mechanisms expand the oxidative stress to media and adventitia layers. Taken together, MMP activity is induced to initiate ECM degradation and proteolysis while VSMC becomes apoptotic to further weaken the vascular wall. NF-κB is also activated, and collagen lysis cannot be balanced by fibroblast. Moreover, the intraluminal thrombosis in adventitia-induced hypoxia causes neovascularization.

13. Diabetes and Aneurysm in Retina Capillary versus Aorta

Retinal capillary aneurysm is a marker of very early diabetic retinopathy (DR), in which diabetes is regarded as a very high risk factor for retinal capillary microaneurysm [78]. Paradoxically, epidemiological data for AAA showed a negative association with diabetes [79–82], although some conflicting data exist [83, 84]. The prevalence of type 2 diabetes in patients with AAA ranged from 6 to 14%, while it was as high as 17 to 36% in the absence of AAAs [85]. It was also found that the growth rate of aneurysms in patients with diabetes is on average lower than in nondiabetics [86, 87]. The mechanism is not yet clearly known. However, despite the negative association, there is a higher mortality rate in diabetes with AAA than nondiabetic patients with AAA [85].

Pericytes covering capillaries have functions similar to smooth muscle cells, that is, maintaining the mechanical stability of capillary structure. Retinal microaneurysm is an

early pathological change in diabetic retinopathy, characterized by the disappearance of pericytes from retinal capillaries. The role of endothelium in DR is not clear. However, recently, it was discovered that hyperglycemia induced PKC activation which had inhibitory effect on eNOS expression in endothelial cells inducing endothelial dysfunction [88]. Moreover, it has been reported that hyperglycemia promotes leukocyte adhesion to the endothelium possibly through upregulation of NF-κB activation which causes apoptosis of endothelial cells [89, 90]. Advanced glycated end products (AGEs) induced by hyperglycemia are bound to its receptors in endothelial cells and pericytes leading to retinal microvessel damage by influencing intracellular functions, such as increased ROS production, vascular stiffness, and cell apoptosis. On one hand, apoptosis of endothelial cells and pericytes in situ position, while futile endothelial cell proliferation in transposing position without pericyte support, both of which lead to microaneurysm formation in the retina.

AAA mice with hyperglycemia demonstrated diminished macrophage infiltration, elastolysis, and neovascularization in their aortic walls [79]. Research has shown that AGE accumulation is negatively correlated with aortic diameter [91] and MMP-2 expression and activity [92]. It is also postulated that medical treatment for diabetes, like metformin, may have a protective effect on AAA. Nevertheless, it seems that retinal microaneurysm formation is different from aortic aneurysms in diabetes. A thorough investigation of the differential correlation between diabetes and aneurysm formation in retinal capillary versus aorta may shed light on a current gap of AAA research.

14. Current Treatments for AAA

At present, surgical repair of expanding arteries or stent installation is the primary measure used to reduce the risk of aneurysm rupture. Whether patients require surgical treatment is dependent on a comprehensive evaluation of many factors, such as the diameter of aneurysm, its growth rate, the presence of symptoms, and life expectancy. Common clinical surgical methods include open surgical repair and endovascular aneurysm repair (EVAR). In EVAR, a tube or stent is implanted into the abdominal aorta cavity to uphold the aortic wall and allow blood flow to pass through the vein. Although, compared with conventional open surgical repair, patients given with EVAR have a short postoperative recovery time and low short-term mortality, there is also no significant difference in long-term (>2 years) mortality.

Patients with a symptomless aneurysm diameter of <40 mm with a low risk of rupture are recommended to be treated with conservative treatments and receive regular reexamination. It is still controversial as to whether patients with an aneurysm size of 40–54 mm should undergo surgery. Some studies have shown that regular color Doppler ultrasound or abdominal aorta CTA examination to monitor the growth of the aneurysm is an effective and safety method. There was no significant improvement to long-term survival rate when these patients underwent early surgical treatment. Therefore, surgical indications of patients with an aneurysm diameter of <54 mm greatly depended upon individual circumstances. The elderly with severe complications, especially, is recommended to receive conservative treatments. Screening of high-risk populations, such as the elderly, smokers, and people with a relevant family history, to detect biomarkers in blood is beneficial for early diagnosis and treatment to reduce the mortality from ruptured aneurysms [93, 94]. It is important that patients who do not have surgical indications should still receive medical treatment in order to limit aneurysm growth.

While surgery is the only measure used to reduce the risk of aneurysm rupture currently, noninvasive medical treatments can be used as adjuvant therapies. A variety of anti-inflammatory, antioxidant, and hemodynamic modulator drugs and MMP inhibitors are currently being studied in order to slow aneurysm growth, reduce the risk of rupture, and improve prognosis as well as mortality after surgery. The growth rate of aneurysm growth is increased in people who smoke, so lifestyle interventions to quit smoking are beneficial in preventing AAA occurrence and delay the aneurysm development [87].

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) reduce the level of ROS products and have anti-inflammatory effects to limit the progress of AAA independent of lipid-lowering effects [95, 96]. Renin–angiotensin–aldosterone system inhibitors, ACEI and ARB, should theoretically slow the arterial dilation and reduce the risk of AAA rupture. While statins and ACEI are well tolerated, a recent meta-analysis on the available data concluded that these drug classes or anti-inflammatory therapies did not influence AAA progression [97]. Although animal studies have demonstrated that beta receptor blockers can inhibit the growth and rupture of aneurysms by influencing hemodynamics, which is beneficial to delay AAA expansion [98], there was no clinical evidence for a beneficial effect of such strategies on AAA progression. On the contrary, evidence was found for growth acceleration in patients taking doxycycline, a MMP inhibitor [99]. This evidence contrasts sharply with the available preclinical data that shows the pharmaceutical interference in aspects of the RAS system, cholesterol metabolism, vascular inflammation, or protease activity effectively alleviates aneurysm formation in rodent disease models [100]. Consequently, although cardiovascular risk management does not influence AAA progression, it is important to realize that risk management is indicated in AAA patients as this group is at an extremely high cardiovascular risk [6].

A pharmacological approach has not been identified that effectively limits AAA progression or the risk of rupture in humans. What has been lacking is a detailed understanding of the mechanisms of AAA initiation and expansion. Studies on the role and significance of vascular endothelial cells in AAA pathogenesis and progression will give a new perspective for a novel target discovery for the prevention of AAA formation and delaying its progression. Statins, anti-RAS drugs, and antioxidants may have some effects on the restoration of endothelial dysfunction but are not effective or specific in eNOS recoupling [30, 101–105]. Folic acid or other drugs by targeting eNOS uncoupling should be developed and trialed in AAA to test their effectiveness [30, 106].

15. Conclusion and Perspective

AAA is a potentially fatal cardiovascular disease, and it will become more and more common as the population ages. The pathological mechanisms of AAA are the result of many factors, including a decrease in VSMCs, MMPs activation, breakdown of ECM, and inflammatory cell infiltration, but the role of endothelial cells cannot be ignored. With the recognition of the importance of endothelial dysfunction in AAA formation as well as validation of the effectiveness of pharmacological therapies in AAA, we may discover a promising strategy for early intervention for high-risk patients or a surgical adjuvant therapy to complement current surgical options in advanced AAA. To this end, the differential role of endothelial function on the development of capillary microaneurysm and AAA in diabetes may underlie a promising solution for AAA.

Consent

The authors notify that there were no potentially overlapping papers in the preparation, submission, or published. The authors of this manuscript have all read and agreed to its content. They are grateful to Professor Jun Fu (Cardiovascular Surgery Department, Wuhan Asian Heart Disease Hospital) for his valuable advices to this paper.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Jingyuan Sun and Hongping Deng contribute equally to this paper.

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Review Article

How AMPK and PKA Interplay to Regulate Mitochondrial Function and Survival in Models of Ischemia and Diabetes

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Adenosine monophosphate-activated protein kinase (AMPK) is a conserved, redox-activated master regulator of cell metabolism. In the presence of oxidative stress, AMPK promotes cytoprotection by enhancing the conservation of energy by suppressing protein translation and by stimulating autophagy. AMPK interplays with protein kinase A (PKA) to regulate oxidative stress, mitochondrial function, and cell survival. AMPK and dual-specificity A-kinase anchoring protein 1 (D-AKAP1), a mitochondrial-directed scaffold of PKA, interact to regulate mitochondrial function and oxidative stress in cardiac and endothelial cells. Ischemia and diabetes, a chronic disease that increases the onset of cardiovascular diseases, suppress the cardioprotective effects of AMPK and PKA. Here, we review the molecular mechanisms by which AMPK and D-AKAP1/PKA interplay to regulate mitochondrial function, oxidative stress, and signaling pathways that prime endothelial cells, cardiac cells, and neurons for cytoprotection against oxidative stress. We discuss recent literature showing how temporal dynamics and localization of activated AMPK and PKA holoenzymes play a crucial role in governing cellular bioenergetics and cell survival in models of ischemia, cardiovascular diseases, and diabetes. Finally, we propose therapeutic strategies that tout localized PKA and AMPK signaling to reverse mitochondrial dysfunction, oxidative stress, and death of neurons and cardiac and endothelial cells during ischemia and diabetes.

1. Introduction

AMPK is a heterotrimeric holoenzyme that consists of a catalytic subunit (α) bound to two regulatory subunits (β and γ). Due to the diverse arrangement of different isoforms of the three subunits of the AMPK holoenzyme, there can exist up to 12 different AMPK holoenzymes in different tissues, which add a layer of complexity AMPK-mediated regulation of critical physiological functions in eukaryotes [1, 2].

AMPK activity is induced by a high ratio of intracellular AMP to ATP levels. AMP directly binds to the regulatory subunit of AMPK to facilitate AMPK phosphorylation via the upstream protein kinase LKB1 [3]. Upon phosphorylation by LKB1, activated AMPK restores intracellular energy levels by inhibiting ATP-consuming biosynthetic pathways and by stimulating catabolic ATP-regenerating processes. Furthermore, AMPK is a redox-sensing kinase that regulates cellular bioenergetics. Indeed, mitochondrial-derived free radicals can activate AMPK through LKB1 without altering the ratio of AMP/ATP [4, 5].

AMPK can be activated via two different mechanisms: (1) phosphorylation of threonine 172 (Thr172) by upstream kinases or via autocatalytic-mediated phosphorylation or (2) by binding of AMP to AMPK. Specifically, LKB1, CaMKKb, or TAK1 can phosphorylate Thr172 within the activation loop of the α-subunit when AMP binds to the

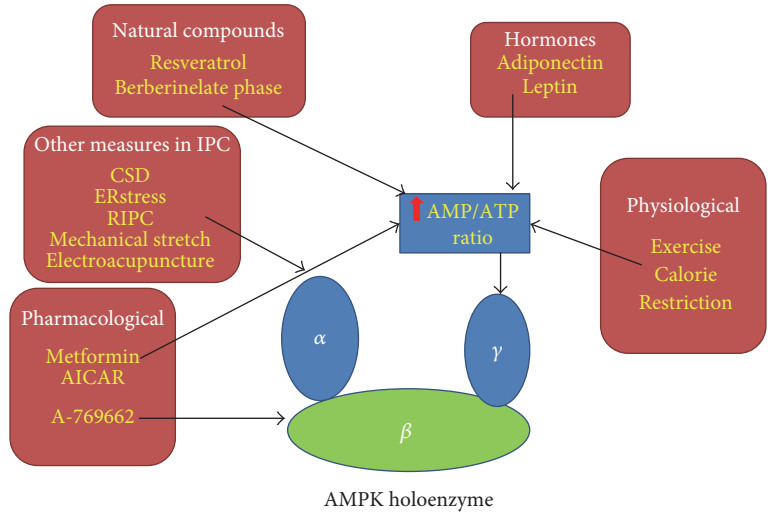

Figure 1: Non-redox-dependent physiological, pharmacological, natural compounds and other conditions that activate AMPK in ischemic preconditioning. This figure highlights some of the well-established and newly discovered AMPK activators or conditions that have benefits in ischemic preconditioning. Pharmacological activators such as metformin and AICAR, some natural compounds, and physiological situations such as exercise and calorie restriction can activate AMPK by increasing the AMP : ATP ratio (shown in the blue rectangle), causing AMP to bind to the γ-subunit. However, a subset of conditions or pharmacological compounds can stimulate AMPK activation in IPC via other mechanisms, such as activated upstream kinases of the α-subunit (shown in the rounded rectangle named "other measures in IPC"), or binding directly to the β-subunit (A769662).

γ-subunit [2, 6–9]. All the aforementioned upstream kinases, as well as levels of AMP induced by acute/chronic stress, can activate AMPK to activate downstream biological functions [10]. Figure 1 and Supplementary Table S1 show ways that AMPK can be activated pharmacologically in a ROS-dependent manner.

The phosphorylation and subsequent activation of AMPK elicit the following physiological functions depending on the type and intensity of toxic insult and oxidative stress: (1) regulation of metabolism and glucose uptake, (2) modulation of protein synthesis and cellular growth by inhibiting the mammalian target of rapamycin (mTOR) pathway, and (3) initiating autophagy during starvation or by specific conditions that induce severe stress [10]. In the context of metabolic stress (metabolic crisis), most cells or organs benefit from activation of AMPK signaling with the exception of brain tissue which has shown modest cytoprotection or detrimental outcomes during ischemia [11]. Activation of AMPK signaling regulates cell metabolism. For instance, AMPK stimulates fatty acid oxidation [12], mitochondrial biogenesis [13–15], glucose transporter type 4 (GLUT4) translocation, and glucose uptake [16–18], while inhibiting protein synthesis, gluconeogenesis, and fatty acid and cholesterol synthesis, [19–21] to increase ATP levels in order to reduce oxidative stress caused by metabolic crisis [22].

Mitochondria not only are regulators of oxidative phosphorylation and calcium homeostasis but also regulate a variety of converging cell death signaling pathways that activate programmed cell death. The mechanisms by which AMPK regulates mitochondrial function and cell survival have recently gained interest, but this area of research is still in its infancy. Recent evidence shows that AMPK has the capacity to regulate cellular bioenergetics, cell death, and mitochondrial structure/function and dynamics by interacting with PKA or, in parallel, during chronic or acute oxidative stress. In this review, we present evidence on the molecular mechanisms by which AMPK and PKA coregulate mitochondrial function and structure in cardiac cells and neurons, two cell types targeted in ischemia and in diabetes, a chronic disease that increases the risk for developing cardiovascular diseases (CVDs) and stroke. Indeed, the current global incidence of obesity and type 2 diabetes has increased in part due to a combination of sedentary lifestyle and high-calorie diets. Secondly, we highlight the importance of how the temporal dynamics and intracellular localization of activated AMPK and PKA holoenzymes play a critical role in regulating oxidative stress and cellular bioenergetics in cardiac cells, endothelial cells, and neurons.

2. AMPK Regulates Mitochondrial Dynamics, Function, and Structure

Since the mitochondria act as major "power plants" of eukaryotes, exploring the interaction between AMPK and mitochondria will shed more insight as to how cells maintain overall energy homeostasis and mitochondrial quality control [23, 24]. In addition, AMPK promotes mitochondrial biogenesis (generation of new mitochondria) in various tissues. For instance, by stimulating PGC1-α and NRF1/2 expression, AMPK participates in parallel to the E3 ubiquitin ligase Parkin to govern mitochondrial biogenesis, presumably as a compensatory response to preserve mitochondrial homeostasis [3, 25–27].

Mitochondrial fission/fusion and movement (trafficking) is regulated by protein phosphorylation imparted by a variety of ser/thr kinases including PKA and AMPK [28, 29].

Recently, there have been an increasing number of studies that show that AMPK regulates mitochondrial structure and function [5, 26, 30–32]. For instance, in neurons, AMPK signaling regulates anterograde transport of mitochondria in the axons of depolarized neurons [33]. In nonneuronal cells, AMPK signaling supports mitochondrial fragmentation (fission) of oxidatively damaged mitochondria by activating the fission modulator dynamin-related protein 1 (Drp1). In support of this concept, a landmark study recently showed that AMPK facilitates mitochondrial fission induced by acute treatment with the complex I inhibitor rotenone. Conversely, U2OS cells deficient for both the α and β subunits of AMPK are recalcitrant to mitochondrial fragmentation induced by mitochondrial-damaging compounds rotenone and antimicyn-A [29]. Mechanistically, AMPK activation promotes mitochondrial fission by phosphorylating mitochondrial fission factor (MFF) and by activating ULK [29, 34].

Cells require a minimum level of high-quality mitochondria to produce the necessary energy to thrive. Oxidatively damaged and effete mitochondria are continuously targeted for autophagolysosomal-mediated degradation via a selective physiological process termed mitophagy [35, 36]. Importantly, an increase in the turnover of mitochondria via mitophagy allows for not only the removal of damaged/effete mitochondria but also for the induction and integration of mitochondrial biogenesis pathways to restore mitochondrial levels [23]. Given that mitochondrial fission facilitates the induction of mitophagy [37], stimulating Drp1-dependent mitochondrial fission via AMPK signaling facilitates the removal of mitochondria via mitophagy [29, 34] in order to maintain a level of healthy mitochondria.

2.1. AMPK Regulates Mitochondrial Quality Control. AMPK is a *bona fide* regulator of mitochondrial dynamics, mitochondrial autophagy, and biogenesis. Indeed, when metabolic stress persists, damaged mitochondria will lead to robust Drp1-dependent mitochondrial fragmentation which can facilitate apoptosis [38, 39]. As mentioned before, activated AMPK triggers mitochondrial fission, at least in part via phosphorylation of MFF, which consequently activates the kinase ULK1 to initiate autophagy. Thus, AMPK may couple mitochondria fission to mitophagy in a continuous feed-forward cycle to maintain the levels of high-quality mitochondria and energy in the cell. A study by Toyama et al., 2016 shed insight on the mechanism by which AMPK activates fission in human U2OS osteosarcoma cells, SV40-immortalized murine embryonic fibroblasts (MEFs), and layer 2/3 cortical pyramidal neurons [29]. On the contrary, other research reports suggest that metformin can inhibit Drp1-mediated mitochondrial fission in endothelial cells of streptozocin- (STZ-) induced diabetic ApoE$^{-/-}$ mice and in adipose tissue of STZ-induced diabetic WT mice [40, 41]. Overall, these studies suggest that AMPK can have opposing effects on mitochondrial fission/fusion, a phenomenon that likely depends on the bioenergetic status and levels of oxidative stress in the cell.

2.2. MFF Level Regulates AMPK-Mediated Fission. One possibility is that the levels of MFF may govern the ability of AMPK to regulate mitochondrial dynamics. MFF was observed to be in low abundance in human umbilical vein endothelial cells (HUVECs) and human vascular smooth muscle cells (HVSMCs) [41]. In addition, AMPK has been found to directly mediate mitochondrial fission via MFF in response to energy stress [29]. Neurons contain intermediate levels of MFF level while MFF is undetectable in astrocytes and brain endothelial cells or other heart or muscle tissues according to the Human Protein Atlas Program. In peripheral tissues, MFF levels are low including myocytes, hepatocytes, endothelial cell, astrocytes, and in renal glomeruli. AMPK regulates mitochondrial fission through MFF in U2OS cells [29]. Interestingly, AMPK may induce mitochondrial fusion in cells that are chronically stressed or in certain pathological conditions. For instance, endothelial cells from streptozotocin- (STZ-) induced diabetic ApoE$^{-/-}$ mice treated with metformin show an inhibition of Drp1-mediated mitochondrial fission [41]. In addition, AMPK activation inhibits high glucose-induced Drp1-mediated mitochondrial fission in epididymal adipose tissue both *in vitro* and in *in vivo* [40]. Supplementary Table S2 gives a comprehensive list of pharmacological compounds that confer protection in cells by activating AMPK in a non-ROS manner (Supplementary Materials online, Table S2). Adipose tissue, another tissue with low levels of MFF, benefits from the inhibition of mitochondrial fission via AMPK activation in hyperglycemic conditions. Overall, it is likely that the levels of oxidative stress and the extent of mitochondrial damage dictate whether AMPK promotes fusion or fission. Future studies that elucidate the mechanisms by which oxidative stress regulates AMPK-mediated mitochondrial fission/fusion are warranted.

During physiological conditions, neurons require a continuous distribution of mitochondria across long distances including dendrites, axons, and synapses in order to meet end-to-end energy requirements, neurotransmission, dendrite development, and efficient Ca^{+2}buffering [42]. Given that AMPK regulates mitochondrial movement in neurons [33], it is conceivable that neurons contain a higher level of MFF to achieve a minimum level of mitochondrial trafficking required in very extensive and vast neuronal networks. Hence, AMPK activation induces diverse effects on mitochondria dynamics which partly depends on levels of endogenous MFF across different tissues.

3. Role of AMPK as a Redox Sensor of Mitochondrial ROS

The mitochondria are the main generators of cellular ATP production via oxidative phosphorylation. However, if protein complexes embedded in the inner mitochondrial membrane or enzymes which catalyze cascade of redox reactions in ETC lose their tight association with the IMM or are damaged, electrons will leak and consequently generate detrimental levels of ROS by interacting with oxygen [43]. For instance, electrons can leak from complex I and react with oxygen to generate superoxide [44, 45]. Indeed, the mitochondria are the primary source of intracellular ROS levels and contribute up to 95% of total ROS levels [46].

Transient and moderate levels of ROS during preconditioning will induce cytoprotective responses by regulating either protein function and/or gene expression [47]. Recent ischemia preconditioning studies show that ROS-mediated activation of AMPK is associated with resistance against ischemia. A myriad of different toxic and physiological stimuli that activate AMPK can exert cytoprotection in models of ischemia. For instance, Supplementary Table S1 shows that hydrogen peroxide, hypoxic conditions, low glucose concentrations, thyroid hormone, and many drugs can activate AMPK in a ROS-dependent manner despite a stable ratio of AMP/ATP [4, 5, 30, 31, 48, 49] (Supplementary Materials online, Table S1).

3.1. Redox-Activation of AMPK Regulates Cell Survival during Ischemia. Sublethal hypoxic and ischemic events (ischemic preconditioning) or some drugs can enhance the tolerance of tissues and organs, to subsequent lethal injury induced by hypoxia, ischemia, and ischemia-reperfusion [50]. The induction of this ischemic tolerance can be achieved by three major approaches: (1) ischemic conditioning, (2) hypoxic conditioning, and (3) chemical conditioning [51]. Interestingly, hypothermic preconditioning exerts a more pronounced cardioprotective effect than ischemic preconditioning [52]. AMPK can be activated by ischemic preconditioning as well [4, 5, 30, 48, 49, 52–54]. In this context, mitochondrial-derived ROS induced by different preconditioning paradigms leads to activation of AMPK and induces resistance to subsequent lethal injury. Mechanistically, ROS scavengers or compound C can both diminish this protection alone suggesting that ROS is a modulator of AMPK-dependent cytoprotection against ischemia [30, 48, 53]. The fact that cells deficient in mitochondrial DNA (ρ0 cells), or cells treated with mito-TEMPO, fail to activate AMPK [4, 5] and abolishes the protective effects induced by ischemic conditioning further corroborates the concept that mitochondrial ROS is required to confer protection against ischemic insults [30].

4. The Diverse Effects of AMPK Activation on Ischemia, Ischemia-Reperfusion, and Preconditioning in the Brain

Some studies have shown that AMPK activation via ischemic preconditioning can prevent injury during ischemia-reperfusion in many organs including the heart, liver, and kidney [49, 55–58]. In contrast, investigators found that acute activation of AMPK prior to lethal ischemia is detrimental to the brain, whereas mild activation of AMPK signaling is beneficial [58–60]. Indeed, the authors of those studies demonstrated detrimental outcomes *in vivo* and *in vitro* as evident by the induction of larger infarct volumes, lower neurobehavioral scores, and decreased cell viability compared to control groups.

It is conceivable that the detrimental effects of acute and prolonged activation of AMPK prior to ischemia depend on both the metabolic status and mitochondrial health of neurons prior to ischemia. Given that neurons consume the majority of glucose (~20% of total glucose) and rely on oxidative phosphorylation to thrive, neurons predominantly utilize glucose as their main substrate for producing energy via oxidative metabolism [61]. However, neurons lack or contain very low levels of the 6-phosphofructo-2-kinase (PFK2) which is required to synthesize fructose-2,6-bisphosphate (F2,6P2), a powerful allosteric activator of PFK1 [62, 63]. Neurons can oxidize fatty acids and utilize amino acids. Hence, when ischemia ensues, p-AMPK will not lead to an increase in glycolysis but increased fatty acid oxidation in neurons, leading to enhanced oxidative phosphorylation to restore the ATP levels. Enhanced AMPK signaling in the brain under anaerobic conditions or hypoglycemia leads to metabolic failure during ischemia with detrimental consequences. Hence, increased AMPK signaling does not consistently protect neurons from ischemia insult as in other cells such as myocyte, hepatocyte, renal cell, endotheliocyte, or even adjacent astrocytes [11, 56, 57, 64].

Over the past decades, other investigators have made considerable efforts to illustrate the role of AMPK in cerebral ischemia. In order to avoid the off-target effects by drugs that activate AMPK, genetic models that ablate the expression for either the α1or α2 catalytic subunit of AMPK have been examined to further elucidate the *in vivo* role of AMPK in cerebral ischemia [65, 66]. Since AMPK activation likely enhances metabolism and survival of astrocytes as in peripheral tissues, increased AMPK activity can provide a favorable bioenergetics environment for neurons via the lactate shuttle [11]. Hence, future studies should explore whether AMPK can be activated specifically in CNS neurons. Indeed, a recent paper shed light on the effects of AMPK activation in neurons *in vivo* [67]. In brief, one study showed that AMPK is not activated in neurons during lethal ischemia phase but only during the ischemic preconditioning phase induced by cortical spreading depression (CSD) prior to the onset of ischemia. In addition, CSD enhances ischemic tolerance to temporary focal ischemia and a significant increase in the levels of phosphorylated α subunit of AMPK occurs 12 h. following CSD [67, 68]. The increased level of phosphorylated α subunit of AMPK was restricted to neurons—neurons predominantly located within the superficial layers of the cerebral cortex—but was not observed in astroglial cells. This observation was further confirmed by Shen et al. 2017 [67].

5. AMPK Can Be a Double-Edged Sword during Ischemia

Based on the aforementioned studies, we can conclude that acute AMPK activation prior to ischemia is protective in peripheral tissues but leads to a detrimental outcome in the brain [49, 56, 57, 64, 69, 70]. As mentioned before, these stark contradictions may be explained by the inherent energy metabolism conditions of neurons prior to the onset of toxic insults (e.g., AMPK increases oxidative phosphorylation during anaerobic conditions if activated during ischemia). However, the extent of activation of the AMPK-MFF-mitochondrial fission axis during ischemia may also contribute to these disparate effects [29, 40, 41]. Hence, future studies in *in vivo* models of ischemia are warranted to understand

the role of the AMPK-MFF-fission pathway on neuronal survival in the context of ischemia.

However, there is a consensus that brief periods of AMPK activation prior to ischemia can enhance neuronal survival whereas sustained activation of AMPK induces cell death [11, 60, 67]. In addition, diverse stimuli that can lead to transient AMPK activation, such as brief glutamate exposure [71] and mild mitochondrial-uncoupling stimuli [72, 73]; brief periods of oxygen glucose deprivation *in vitro* [74]; or brief and intermittent blood vessel occlusion cycle *in vivo* [60], and CSD [67] can enhance tolerance to ischemia in an AMPK-dependent manner. AMPK activation in this manner prevents neurons from degenerating during ischemia or ischemia/reperfusion by initiating autophagy [67, 74], inducing translocation of glucose transporter 3 (GLUT3) [71], and promoting higher mitochondrial membrane potential to maintain Ca^{+2} homeostasis [75] or directly decrease AMPK levels in lethal ischemia [60]. Therefore, AMPK is an enticing target for eliciting neuroprotection via ischemia preconditioning in the brain despite its proapoptotic role during lethal ischemia. This concept is further elaborated below in Section 8. However, this phenomenon is just another example of the importance of ischemic preconditioning and reinforces the concept that "what does not kill you will make you stronger", quoted by Annalisa Carlucciby Nietzsche.

6. D-AKAP1 Interacts with AMPK to Regulate Survival: Implications for Ischemia/Diabetes

D-AKAP1 (AKAP140/149 and other splice variants AKAP121, sAKAP84) is a protein scaffold that targets PKA to the outer mitochondrial membrane (OMM) to phosphorylate the proapoptotic protein BAD and the profission protein Drp1 [76, 77] to induce mitochondrial fusion and stabilize mitochondrial networks, a phenomenon that is associated with enhanced neuroprotection against toxic insults [28, 77, 78].

Recent evidence suggests that D-AKAP1 and AMPK interact to regulate mitochondrial function and structure. Indeed, D-AKAP1 is a substrate of AMPK. By using targeted *in vitro* AMPK screens and phosphorylation prediction algorithms, one recent study elegantly showed that D-AKAP1 is a substrate of AMPK in skeletal muscle cells [79]. The physiological effects of AMPK-mediated phosphorylation of D-AKAP1 include an increase in oxidative phosphorylation and mitochondrial-mediated β oxidation of lipids in L6 myoblasts. Mechanistically, AMPK phosphorylates D-AKAP1 in S103 to maintain mitochondrial respiration and transmembrane potential [79]. However, it remains to be seen whether AMPK phosphorylates D-AKAP1 in other tissues. Moreover, it remains to be known whether AMPK-mediated phosphorylation of D-AKAP1 affects mitochondrial dynamics and quality control. In addition, it is conceivable that AMPK uncouples PKA from D-AKAP1 to promote mitochondrial fission and mitophagy. Indeed, there is rationale and evidence that other signaling pathways can uncouple PKA from D-AKAP1 to promote fission. For instance, PINK1, a ser/thr mitochondrial kinase mutated in recessive forms of Parkinson's disease, triggers the displacement of PKA from D-AKAP1 during toxic insults that damage mitochondria and thereby ensures that fission of damaged mitochondria promotes mitophagy [80]. Hence, during oxidative stress, it is conceivable that AMPK-mediated phosphorylation of D-AKAP1 may limit the accessibility of PKA to D-AKAP1, presumably to allow AMPK to phosphorylate MFF as a feed-forward mechanism to promote mitochondrial fission. In summary, we raise the possibility that AMPK and D-AKAP1/PKA participate in a "tug of war" to regulate mitochondrial fission/fusion. For instance, increased AMPK signaling may induce mitochondrial fission by opposing D-AKAP1/PKA. The D-AKAP1-PKA-Drp1signaling axis promotes mitochondrial fusion whereas the AMPK-mitochondrial fission/fusion signaling axis leads to mitochondrial fragmentation and subsequent activation of mitophagy during toxic stress in neurons, both pathways leading to increased cell survival and mitochondrial homeostasis. However, in the absence of D-AKAP1/PKA signaling during oxidative stress, AMPK can act as a beneficial, compensatory signaling pathway to exert cytoprotection as elaborated below.

There is clear evidence that AMPK can converge at the mitochondrion to enhance protective D-AKAP1/PKA signaling. As reiterated, PKA-mediated phosphorylation of Drp1 via D-AKAP1 promotes mitochondrial fusion and prevents the activation of apoptosis in cells [28]. However, in models of diabetes and chronic stress, PKA-mediated phosphorylation of Drp1 is decreased, leading to mitochondrial fission [40, 81]. Treating cells with AMPK activators such as metformin or AICAR restores mitochondrial interconnectivity by enhancing PKA-mediated phosphorylation of Drp1 [49, 53]. However, additional studies are warranted to unveil the mechanism by which AMPK signaling interplays with mitochondrial PKA in models of ischemia and diabetes.

Beyond the mitochondrion, AMPK and PKA have been shown to cross-talk and interact in a feed-forward manner. For instance, treating vascular smooth muscle cells with AICAR not only stimulates AMPK signaling but also enhances PKA signaling [82]. However, the mechanisms by which these two holoenzymes interact, and whether this interaction occurs in cardiac cells, remain to be elucidated in future studies.

D-AKAP1 is a cardioprotective protein scaffold of PKA. D-AKAP1 is rapidly targeted for proteolytic degradation during oxidative stress via the E3 ligase Siah2 during ischemia [83, 84]. Chronic depletion of endogenous D-AKAP1, as evident in D-AKAP1 knockout mice, show aberrant mitochondrial structure as assessed by electron microscopy, increased ROS production, and reduced mitochondrial function following myocardial infarction (MI). These alterations were associated with robust cardiac mitophagy and apoptosis. Interestingly, reductions in D-AKAP1 levels are correlated with increased infarct size following myocardial infarction in D-AKAP1$^{-/-}$ mice subjected to ligation of the left coronary artery [84]. However, this study did not address whether elevated mitophagy observed in D-AKAP1 knockout mice subjected to MI is phenocopied by AMPK activation. Consistent with this study, another study showed that

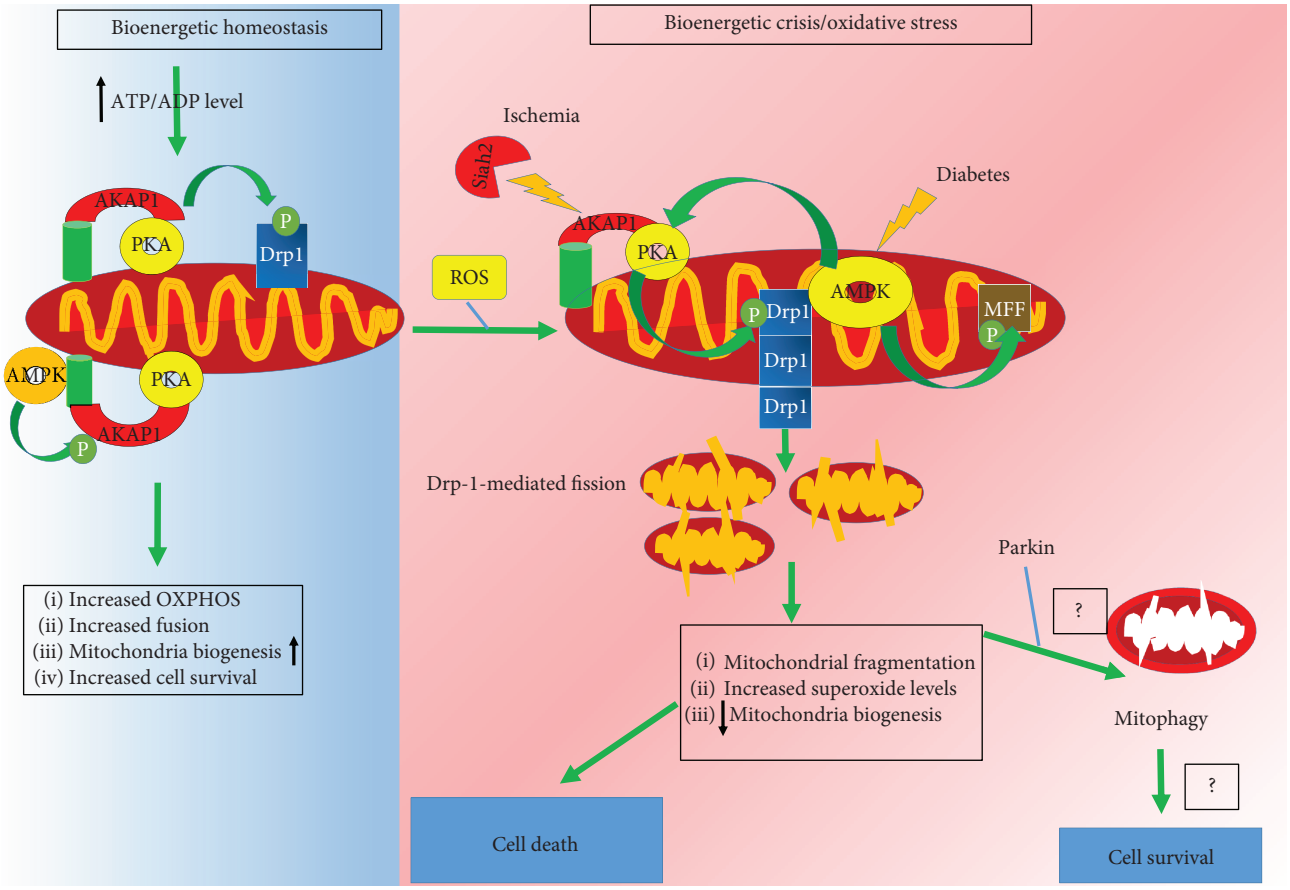

Figure 2: Model on how AMPK and D-AKAP1/PKA cooperate to regulate mitochondrial structure/function under physiological conditions and during oxidative stress induced by diabetes and ischemia. During homeostasis (indicated in shaded blue), D-AKAP1/PKA and AMPK regulate mitochondrial structure and function to maintain a high ATP/ADP ratio. D-AKAP1/PKA phosphorylates Drp1 at the OMM to inhibit its fission activity and, thereby, promote mitochondrial fusion and maintain oxidative phosphorylation. Concomitantly, AMPK phosphorylates D-AKAP1 leading to stable mitochondrial bioenergetics and structure through an unknown molecular mechanism. These posttranslational events lead to enhanced mitochondrial biogenesis and cell survival. On the other hand, acute or a transient increase in the level of oxidative stress (indicated in shaded red) leads to decreased kinase signaling (uncoupling of PKA from D-AKAP1) and decreased mitochondrial oxidative phosphorylation and mitochondrial dysfunction (decreased transmembrane potential), ensuing mitochondrial damage. AMPK phosphorylates MFF to promote mitochondrial fission, a cellular event that is associated with increased mitophagy. However, is not known whether increased AMPK-mediated fission enhances mitophagy or increased cell survival (conceptual gaps indicated by question marks). On the other hand, conditions that promote ischemia or chronic high levels of oxidative stress, as observed in models of diabetes and CVDs, leads to rapid degradation of endogenous D-AKAP1 through Siah2 (hypoxia), decreased AMPK signaling (diabetes models), increased superoxide levels, and decreased compensatory responses to replenish high quality mitochondria and eventual cell death.

a rat model of cardiac hypertrophy depletes D-AKAP1 levels in heart tissue, molecular pathology that is associated with mitochondrial dysfunction [85]. Furthermore, cardiac hypertrophy led to a decrease in endogenous levels of D-AKAP1 via downregulation of nuclear-localized cAMP signaling pathways and significantly increased ROS production [85].

Autophagy may be protective during ischemia, whereas it may be detrimental during reperfusion [86–88]. Thus, D-AKAP1 and AMPK stimulate mitochondria biogenesis and coregulate mitochondrial fission/fusion. Therefore, the interplay of these two proteins during oxidative stress may explain how these two ser/thr kinase govern ischemic preconditioning. Hence, we propose that maintaining normal levels of D-AKAP1 is necessary to preserve a pool of high quality and healthy mitochondria while low levels of D-AKAP1 may stimulate mitophagy, presumably via AMPK activation, of damaged mitochondria following ischemic conditioning (Figure 2).

7. The Dual Roles of AMPK Signaling in Diabetes

AMPK has dual effects on mitochondrial function and structure, which likely depends on the levels of oxidative stress of cells. Under physiological conditions, AMPK promotes mitochondrial fission, presumably to stimulate mitophagy as a protective mitochondrial quality control mechanism [29]. However, in chronically stressed cells, AMPK confers cytoprotection, presumably by promoting mitochondrial fusion, mitochondrial biogenesis, eliciting

antioxidant responses, and restoring mitochondrial function [29, 89, 90]. Another study showed that enhancing AMPK signaling promotes mitochondrial fusion and reduces cell death caused by ischemia/reperfusion *in vivo* and *in vitro* [30].

Diabetes increases the risk for damaging endothelial cells and heart tissue. In addition, AMPK signaling is protective in several *in vivo* models of diabetes [91]. For instance, *in vivo* rodent models of diabetes exhibit decreased AMPK-mediated signaling (Thr-172 phosphorylation), in liver and kidney tissues [26]. Therefore, reduced AMPK signaling is associated with an inability of cells to mount necessary AMPK-mediated responses to compensate for the loss of energy and mitochondrial dysfunction (Figure 2). In cell cultures of diabetes, enhancing AMPK signaling can protect cells against hyperglycemia and hypoglycemia [91]. For instance, cell stress induced by hyperglycemia can elicit Drp1-mediated mitochondrial fission and increased mitochondrial superoxide, cytopathology that is reversed by elevating AMPK signaling [92]. In addition, pancreatic β cells maintained in low levels of glucose exhibited an increase in superoxide levels, decreased mitochondrial oxidative phosphorylation, and robust phosphorylation of AMPK and of AMPK substrates [31]. However, the physiological implications for AMPK activation in pancreatic β cells remain to be elucidated in this context.

Pharmacological activators of AMPK are cardioprotective in models of diabetes.

Indeed, the AMPK activator metformin efficiently reduces the steady-state levels of mitochondrial superoxide and mitochondrial fission in endothelial cells derived from streptozocin-treated rats, a well characterized *in vivo* model for diabetes [26]. The protective effects of metformin require AMPK activation as transfecting hyperglycemic HUVECs with a constitutively active mutant of AMPK phenocopies the ability of metformin for blocking Drp1-mediated mitochondrial fission and Drp1-mediated increased levels of mitochondrial superoxide [41]. Another study showed that rats treated with streptozocin for four weeks showed a marked reduction in the levels of superoxide, decreased mitochondrial biogenesis, decreased oxidative phosphorylation, and phosphorylation of AMPK in the heart and the kidney [26]. Pharmacologically cotreating streptozocin-treated mice with the AMPK activator AICAR restores AMPK signaling, reverses mitochondrial pathology, restores mitochondrial oxidative phosphorylation, and reverses kidney pathology, further supporting the concept that activating AMPK signaling is protective in models of diabetes [26] (Supplementary Materials online, Table S2). In another study, treating streptozocin/ApoE$^{-/-}$ mice with metformin was able to reduce mitochondrial fragmentation [41].

Physiological activation of AMPK activation can protect the heart from ischemia by upregulating glucose uptake and energy-generating glycolytic pathways as well as enhancing fatty acid oxidation. Specifically, phosphorylation of phosphofructokinase [63] by AMPK can promote the generation of ATP *via* glycolysis [63]. However, downstream ischemia-protective pathways activated by AMPK are blunted in type 2 diabetes. In this context, heart tissue exhibits little flexibility to compensate for energy loss in a similar manner to what neurons undergo during ischemia (Figure 2). A high body mass index and obesity are risk factors for developing CVDs and diabetes. Interestingly, endogenous mRNA levels of D-AKAP1 and of type II regulatory subunit of PKA (PKA/RIIβ) have been observed to be decreased in adipocytes and subcutaneous adipose tissue of obese individuals [93, 94]. Although is not known whether D-AKAP1 levels are downregulated in tissues of diabetes individuals, it is worth noting that D-AKAP1 is transcriptionally regulated by PPRγ in a PKA-dependent manner [93]. Therefore, given that PPRγ-mediated signaling is impaired in type II diabetes, these observations suggest that decreased expression of D-AKAP1 and PKA/RIIβ—proteins involved in lipolysis and mitochondrial metabolism—may contribute to the pathology in CVDs and type II diabetes subjects with a high BMI. In addition, patients with type 2 diabetes (T2D) are highly susceptible to developing CVD and restoring normoglycemia alone is insufficient for reducing the risk of CVDs in T2DM [95] suggesting that other therapeutic strategies need to be developed to reduce the onset of cardiovascular complications. In support of this concept, several known antidiabetic drugs in clinical work are used to reduce the incidence of diabetes-related CVDs such as metformin, thiazolidinediones (TZDs), and statins. Indeed, Cilostazol has been shown to restore AMPK activation and exert cardio- and vasculoprotective actions in *in vivo* or *in vitro* [96–102].

8. Therapeutic Perspectives

Diabetic individuals are highly prone to experiencing strokes, minitransient ischemic episodes, and other cerebral vascular complications [91]. As mentioned before, there is a consensus that AMPK is protective in models of diabetes. Given that the levels of AMPK signaling are severely compromised in diabetic tissues [26, 103], normalizing AMPK activity—as induced by metformin—continues to be an enticing therapeutic strategy for treating diabetes. On the other hand, it is not clear whether eliciting AMPK signaling is protective in models of ischemia. Some studies have shown that AMPK is protective during the ischemic phase in mice subjected to ischemia/reperfusion paradigms [104]. *In vitro*, pharmacologically pretreating tissues with AMPK activators can protect in cell culture and *in vivo* models of ischemia [15, 105, 106] (Supplementary Materials online, Table S2). Conversely, one *in vivo* study showed that inhibitors of AMPK activity increased protection against brain damage following ischemia [107]. Hence, future studies are warranted to further identify specific "windows" of opportunity by which AMPK confers robust neuroprotection during ischemia.

8.1. D-AKAP1: An AMPK Substrate with Therapeutic Applications. Overall, the aforementioned published data suggests that PKA and AMPK converge in the mitochondrion to enhance cytoprotection against ischemia. However, as mentioned before, how mitochondrial PKA (D-AKAP1/PKA) and AMPK cooperate to regulate survival of neurons or cardiac cells depends on the oxidative status of cells. During chronic stress, which may decrease PKA signaling

in mitochondria, AMPK may serve to promote mitophagy. However, during physiological conditions, both kinases may cooperate to maintain mitochondrial function and survival. Consistent with the concept that AMPK and mitochondrial PKA cooperate to maintain cell survival during ischemia, one study demonstrated that PKA-mediated phosphorylation of Drp1 and PKA-mediated mitochondrial fusion during nitrite-preconditioning conditions exert cytoprotection of cardiac myocytes against ischemia *in vivo* and *in vitro* [30]. Nitrite-induced PKA-mediated protection of cardiac cells against ischemia requires AMPK activity and mitochondrial ROS [30]. Hence, given that AMPK signaling is blunted in diabetes, these studies suggest that therapeutic interventions that activate mitochondrial PKA can be beneficial to prevent the onset of ischemia in diabetes.

D-AKAP1 is highly sensitive to proteolytic degradation via the E3 ubiquitin ligase Siah2 during ischemia [83, 108]. Given that D-AKAP1 robustly and rapidly undergoes Siah2-mediated degradation during ischemia, it is conceivable that small molecular compounds that can coactivate PKA or stabilize endogenous levels of D-AKAP1 may confer significant protection during ischemia. There is experimental evidence to support this notion. For instance, Siah2 knockout mice exhibit little heart pathology after being subjected to ischemia reperfusion (e.g., myocardial infarct size) [108]. Cells treated with diffusible cyclic AMP analogues can increase the expression of D-AKAP1 in cell culture studies [109]. Therefore, it is conceivable that other PKA activators (e.g., forskolin) could be employed to increase D-AKAP1 *in vivo* to prevent the degradation of D-AKAP1 during ischemia.

There is significant controversy on the consequences of global activation of PKA on cell survival during ischemia. For instance, overt PKA activation exacerbates pathology in heart tissue during ischemia. Indeed, excessive β-adrenergic receptor activation leads to activation of protein kinase A (PKA), leading to increased opening of the L-type calcium currents and a subsequent increase in cytosolic calcium levels, the latter being potentially harmful to cardiac tissue [110, 111]. On the other hand, treating ischemic heart tissue with H89, an inhibitor of protein kinase A, promotes postischemic cardiac contractile recovery and reduces infarct size [112]. In neurons, the binding of cyclic AMP to the regulatory subunit of PKA is rapidly inhibited during the acute phase of cerebral ischemia, leading to reduced neuronal survival [113–115]. Hence, in this case, enhancing PKA signaling prior to ischemia may prove beneficial.

In addition, activation of global PKA activity can negate the protective effects of AMPK in different models of chronic stress and degeneration. For instance, while different studies have shown that PKA governs AMPK-mediated mitochondrial biogenesis and cytoprotection [25, 116], cytosolic PKA has been shown to oppose various physiological effects of AMP kinase in insulin-signaling cells and blocks the ability of metformin for decreasing glucose levels in primary hepatocytes [117, 118].

Given that D-AKAP1 is a substrate of AMPK, it is conceivable that compounds that elicit AMPK-mediated phosphorylation of D-AKAP1 are a better therapeutic option as opposed to using global PKA activators (e.g., forskolin) for conferring neuroprotection during ischemia. On the other hand, AMPK-activating compounds such as AICAR increases PKA signaling *in vitro* [82]. Hence, it remains to be seen whether cotreatment of heart cells or neurons with AMPK and PKA-stimulating compounds exert an additive, protective effect during ischemia. Alternatively, compounds that increase the endogenous levels of D-AKAP1 can offer cytoprotection in models of ischemia. Experimentally induced cardiac infarct depletes D-AKAP1 levels, molecular pathology that is associated with mitochondrial dysfunction [85]. In that study, cyclic AMP analogues were used to increase endogenous D-AKAP1 levels to protect cardiac cells from ischemia insult. To this end, it is conceivable that pharmacological compounds that increase D-AKAP1 levels can be used in hypertensive individuals with left ventricular hypertrophy. Therefore, we raise the possibility that D-AKAP1/PKA and AMPK are novel therapeutic targets for treating ischemia. However, future studies are warranted to elucidate whether single or dual pharmacological activation of mitochondrial PKA or AMPK can confer protection during ischemia.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Supplementary Materials

Table S1: Different conditions that induce AMPK activation through ROS. Table S2: Other conditions that activate AMPK in a non-ROS-mediated mechanism. *(Supplementary Materials)*

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Clinical Study

Low-Level Laser Irradiation Exerts Antiaggregative Effect on Human Platelets Independently on the Nitric Oxide Metabolism and Release of Platelet Activation Markers

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Aim. The goal of the study is to develop a model allowing to investigate precisely the effect of low-level laser therapy (LLLT) on platelet aggregation and to verify the hypothesis regarding the role of the nitric oxide (NO) bioavailability and platelet activation markers in modulating platelet aggregation. *Methods*. A total of 41 healthy volunteers at the age of 21–45 years were investigated. At first, platelet aggregation in response to three agonists (TRAP, ADP, and collagen) was evaluated following previous exposure to different doses of laser radiation ($\lambda = 662$ nm) to assess the dose-response effect. Subsequently, plasma levels of platelet activation markers (PF4—platelet factor-4 and sP-selectin) as well as the substrate for nitric oxide synthase, L-arginine, and its competitive inhibitors (ADMA—asymmetric dimethylarginine and SDMA—symmetric dimethylarginine) were measured. *Results*. All doses of laser irradiation significantly reduced the aggregation. However, the most pronounced effect was observed for 19.7 J/cm^2. No significant differences in the levels of platelet activation markers nor in the nitric-oxide-metabolic-pathway compounds between analyzed groups were noted. *Conclusions*. We have demonstrated in the established *in vitro* experimental model that the LLLT in a reproducible manner decreases the whole blood platelet aggregation regardless of the NO bioavailability or changes in the platelet activation markers.

1. Introduction

Numerous studies have shown that the low level laser therapy (LLLT) modulates biological processes in human cells. The most important changes in cellular metabolism include increased activation of intracellular enzymes involved in the respiratory chain and increased synthesis of DNA and RNA as well as regulation of apoptosis [1]. As a result, the low-energy laser radiation has found many applications in a routine clinical practice.

Growing body of attention within the last few years has been paid to LLLT as part of cardiovascular therapy. Recently, we have shown that intravascular irradiation with low-energy laser during percutaneous coronary intervention (PCI) decreases the magnitude of restenosis and may modulate the inflammatory process in vascular wall [2, 3]. Although this method has been demonstrated to be a safe therapeutic option, the effect of LLLT on platelet activity remains unclear. The results of studies carried out so far have been inconsistent. Some of them suggest

increased platelet activity following exposure to low-energy laser. Hoffman and Monroe showed that LLLT can enhance the platelet activation [4]. On the other hand, Mohan et al. [5] noted decreased platelet responsiveness following the LLLT. Similar results were observed by Eldar et al. [6] and Brill et al. [7].

Several factors are postulated to modify platelet activity and inflammatory response, among which nitric oxide (NO) is one of the best known [2–7]. The low-energy laser irradiation exposure increases the production of NO in some experimental models conducted *in vitro* and *in vivo* [8, 9]. Nevertheless, the exact mechanism of this phenomenon is unknown [8, 10]. Nitric oxide reduces platelet adhesion and aggregation [11].

Hence, we intended to investigate whether NO is a potential transmitter of LLLT modifying platelet activity. In order to explore the impact of LLLT on platelet activation, the plasma levels of the PF4 and sP-selectin were measured in the samples both at baseline and following the laser irradiation.

2. Material and Methods

All experiments were conducted and approved in accordance with the guidelines of the local Bioethics Committee and adhered to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects (revised November 13, 2001, effective December 13, 2001), and all patients enrolled had signed the informed consent to participate in the study.

Only healthy volunteers aged 21 to 45 years were enrolled in the study. The subjects did not use drugs that will potentially affect the obtained results, such as acetylsalicylic acid and other nonsteroidal anti-inflammatory drugs (grace period was 10 days), and hormonal contraception (washout period of 3 months). Patients taking drugs that affect the metabolism of nitric oxide, including phosphodiesterase inhibitors, dietary supplements containing L-arginine, and nitrates, were also excluded from this experiment.

The study was divided into two phases. The first stage aimed at determining the radiation dose causing the most potent biological effect (analysis of the dose-response curve). It was evaluated by changes in the whole blood platelet aggregation induced by selected agonists (thrombin receptor activating peptide (TRAP-test), ADP (ADP-test), and collagen (COL-test)). Five different doses of irradiation were applied. Immediately after donation, the whole blood (500 μl) was moved to special plastic dots with 20 mm diameter (used in everyday practice to perform the blood group tests) and subsequently irradiated using 5 different energy doses. What is important, during radiation exposure, a control (nonirradiated whole blood obtained from the same patient) was also kept in the dots and next aggregation was measured using an impedance aggregometer (Multiplate® analyzer, Dynabyte Medical, Germany)—paired analyses. In the first stage of the study, the investigated group constituted nine subjects (5 male and 4 female at mean age of 28.9 ± 4.7 y)—nine paired analyses.

In the second stage, only the most effective radiation dose was applied. The same agonists were used, and the same conditions of irradiation were applied which was followed by additional biochemical analyses conducted including platelet activation markers and metabolites of nitric oxide metabolic pathway (see below). Afterwards, platelet aggregation was performed under the same conditions as specified above. This part of the study involved 41 participants—20 women and 21 men (at the mean age of 27.5 ± 7.2 y).

2.1. Blood Collection. For platelet aggregation testing, the whole blood was collected into the Sarstedt S-Monovette Hirudin tube (number 04.1944.001). Unlike citrate or heparin, hirudin prevents blood clotting by direct thrombin inhibition, enabling thrombocyte function diagnostics in a native condition.

In order to obtain plasma for determination sP-selectin, PF4, L-arginine, ADMA, and SDMA in the second part of the experiment, blood was collected in the Sarstedt S-Monovette (1.6 mg EDTA/ml blood) tube and within 30 minutes after the collection, it was centrifuged at $1000 \times$g for 15 min at 4°C and stored at −20°C until analysis.

2.2. Laser Source and Its Validation. In this study, a semiconductor laser (Optel®, Poland) was used, where the diode was optically connected ("pigtailed") with optical fiber. The optical system was placed at a 15 cm distance from the blood samples. The diffusion system located at the distal end of the optic fiber caused scattering of the laser beam to a circle with a diameter of 2 cm. The wavelength of radiation emitted by the device was 662 nm, and the setup irradiance was 0.050 W/cm^2 for all radiant exposure doses. During the first stage of the experiment, we planned to use five different doses growing in geometric progression, namely, 2 J/cm^2, 4 J/cm^2, 8 J/cm^2, 16 J/cm^2, and 32 J/cm^2.

Due to significant methodological differences in studies conducted so far, we decided to validate the experimental model. Before irradiation of blood samples, the validation of exact parameters of laser radiation reaching the test samples was performed. For this purpose, we used a manual meter PMD100D (Thorlabs Ltd., New Jersey, USA). Measurements were conducted at the same place as the blood samples were irradiated. The results showed that the exact measured radiant exposure doses were 2.4 J/cm^2, 4.9 J/cm^2, 9.9 J/cm^2, 19.8 J/cm^2, and 39.5 J/cm^2.

An irradiance of laser was also validated and reached 0.053 W/cm^2. In order to verify the wavelength of the radiation emitted by the semiconductor laser source, we used a Yokogawa-aq-6370c Optical Spectrum Analyzer (Japan). The analysis of wavelength spectrum, which was dedicated for the experiment, confirmed a monochromatic light (with a peak for a wavelength of $\lambda = 662.3$ nm).

2.3. Aggregation. The whole blood aggregation was measured using an impedance aggregometer (Multiplate analyzer, Dynabyte Medical, Germany). During the analysis, the sample-reagent mixture was stirred with a discardable PTFE- (polytetrafluoroethylene-) coated magnetic stirrer (800 U/min). Preheated to 37°C saline (300 μl) was placed

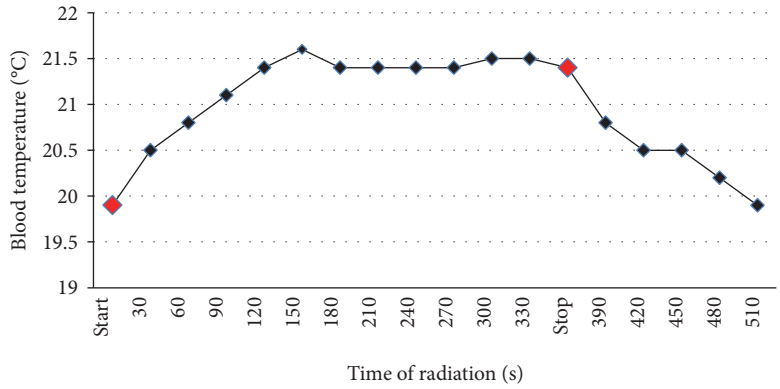

Figure 1: Thermal effect of the blood sample laser irradiation. The maximal increase was observed from 150th second from the beginning of the irradiation procedure (start), and the temperature recovered to the baseline values within the 150 s following cessation of irradiation (stop).

into the test cells with the addition of anticoagulated whole blood (300 μl) and stirred at 37°C; the measurements were started by adding 20 μl of the appropriate agonist: thrombin receptor activating peptide (TRAP-test, Cat. number 6675883190, Roche Diagnostics) at a final concentration of 10.5 μM, ADP (ADP-test, Cat. number 6675794190, Roche Diagnostics) at a final concentration of 3.2 μM, and collagen (COL-test, Cat. number 6675832190, Roche Diagnostics) at a final concentration of 1.05 μg/ml. The change in impedance by the adhesion and aggregation of platelets on the electrode wires was continuously detected. The mean values of the two determinations are expressed in arbitrary units (AU).

2.4. Platelet Activation Markers. Plasma concentrations of sP-selectin/CD62P at baseline as well as following the LLLT were determined by a sandwich enzyme immunoassay technique, using commercial ELISA kits (Cat: BBE6, R&D Systems Europe Ltd., UK) with a sensitivity of 0.5 ng/ml, according to the manufacturer's instructions. Optical density 450/620 nm was measured with a BioTek Absorbance Microplate Reader with software Gen5. The intra-assay CV was less 6%, and interassay CV was less 10%.

In order to measure the concentration of PF4 in plasma (at baseline as well as following the LLLT), we used the commercial test human PF4 ELISA kits (Cat: ELH-PF4, RayBiotech Inc., Georgia, USA) with a sensitivity of 20 pg/ml, according to the manufacturer's instructions. Optical density 450 nm was measured with a BioTek Absorbance Microplate Reader with software KC4. The intra-assay CV was less 10%, and the interassay CV was less 12%.

2.5. The Metabolites of Nitric Oxide Metabolic Pathway. Evaluation of LLLT-dependent changes in the nitric oxide availability was analyzed by assessing the PRMT-L-Arg/ADMA-DDAH axis. Plasma levels of L-arginine (a substrate for NO synthase) and its methyl derivatives (asymmetric and symmetric dimethylarginine—ADMA, SMDA, competitive inhibitors of the nitric oxide synthase) were measured by a high-performance liquid chromatography (HPLC). Plasma samples and standards extracted a solid-phase extraction cartridge with SCX50 columns (Varian Inc., USA). Eluates were derivatized with *o*-phthaldialdehyde (OPA) and separated by isocratic reversed-phase chromatography on a Symmetry C18 column (150 × 4.6 mm, 5 μm particle size; Waters Corp., USA). Potassium phosphate buffer (50 mM, pH 6.6) containing 12% acetonitrile was used as the mobile phase at a flow rate of 1.1 ml/min. Fluorescence detection was performed at the excitation 340 nm and emission 450 nm wavelengths. The test was performed on a computer controlled by Star Chromatography Workstation software (version 6.3); the device was made by Varian (New York, USA).

2.6. Thermal Effect. In order to exclude the importance of the possible thermal effect of LLLT on the aggregation results, we decided to measure the temperature increase during irradiation. Measurements were made using an AX5002 AXIOMET Temp Company (Sweden) with measurement accuracy of ±0.1°C. Temperature measurements had been carried out throughout the period of exposure and continued until blood temperature returned to the baseline values (Figure 1).

We observed minimal thermal effect during the laser irradiation. After radiation dose used in the main stage of the study (19.8 J/cm^2), the absolute increase of blood temperature was 1.5°C from the beginning of the irradiation (start) (Figure 1). It returned to preexposure level after 150 seconds following cessation of irradiation (stop) (Figure 1).

2.7. Energy Dose. Based on available literature we selected five different radiant exposure doses to irradiate blood (2.4 J/cm^2, 4.9 J/cm^2, 9.9 J/cm^2, 19.8 J/cm^2, and 39.5 J/cm^2). Immediately after irradiation, aggregation tests were carried out.

2.8. Statistical Analysis. The data is expressed as mean ± SD. Normality of distribution was verified with Shapiro-Wilk test and the unity of variance by a Levene's test, as appropriate. In the case of quantitative parametric variables, we used Student's *t*-test.

In the case of nonparametric variables, Mann–Whitney's analysis was performed, as appropriate. The analyses were conducted using the Statistica 10.0 (StatSoft) software.

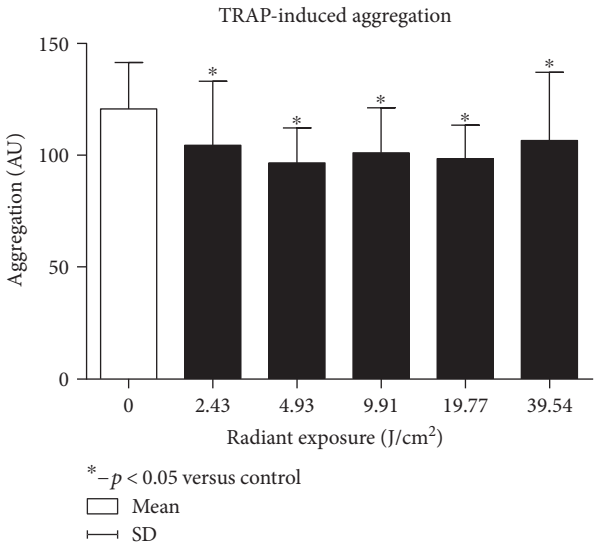

Figure 2: Dose-response effect in the platelet TRAP-induced aggregation.

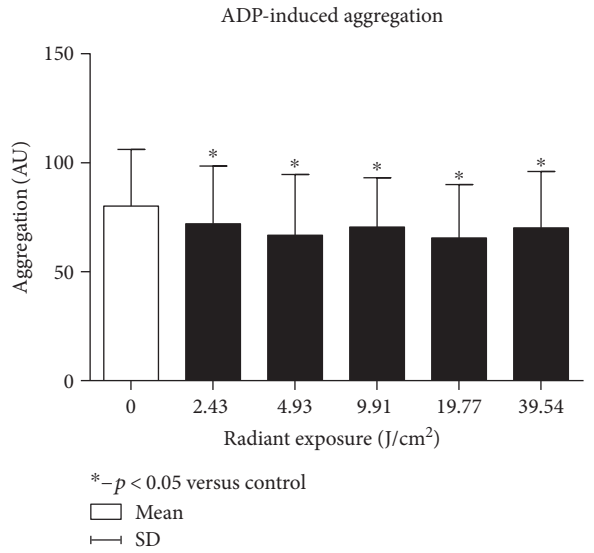

Figure 3: Dose-response effect in the platelet ADP-induced aggregation.

3. Results

The first phase of the experiment proved that each of the radiation dose applied caused a significant decrease in platelet aggregation when compared to the nonirradiated control group, regardless of the agonist administered.

For TRAP, the greatest decrease was observed for a dose of 4.9 J/cm^2. Statistically significant but less pronounced decrease in aggregation was also obtained for doses of 2.4 J/cm^2, 9.9 J/cm^2, 19.8 J/cm^2, and 39.5 J/cm^2 (Figure 2).

The greatest decrease in aggregation for collagen and ADP was observed for radiant exposure dose of 19.8 J/cm^2 ($p = 0.0072$ for collagen and $p = 0.0108$ for ADP, resp.) (Figures 3 and 4). No statistically significant differences in aggregation response between the various doses of radiation were observed. Only greater antiaggregatory effect was observed for a dose of 9.9 J/cm^2 than 39.5 J/cm^2 for ADP as an agonist. Due to the fact that the greatest biological effect was obtained with a dose of 19.8 J/cm^2, we used that one in the second phase (Figures 3 and 4).

The second phase of the study involved 41 young healthy participants—20 women and 21 men. For all the agonists (ADP, TRAP, and collagen), the aggregation results following LLLT were statistically significant in comparison to the not irradiated control (not irradiated) sample (Table 1).

In order to verify the involvement of potential molecular mechanisms by which the LLLT affects platelet aggregation, we analyzed changes levels of platelet activation markers as well as the levels of the nitric oxide bioavailability markers (L-arginine—a substrate for the nitric oxide synthase, and its competitive inhibitor—ADMA as well as SDMA characterized by less pronounced inhibitory properties) using energy dose of 19.8 J/cm^2. There were no statistically significant differences in the concentrations of L-arginine, ADMA, nor SDMA between the two analyzed groups irradiated one and the control without LLLT (Table 1). Similar results were observed regarding plasma platelet activation markers—no statistically significant differences in the levels of PF4 and sP-selectin between irradiated and nonirradiated blood were observed (Table 1).

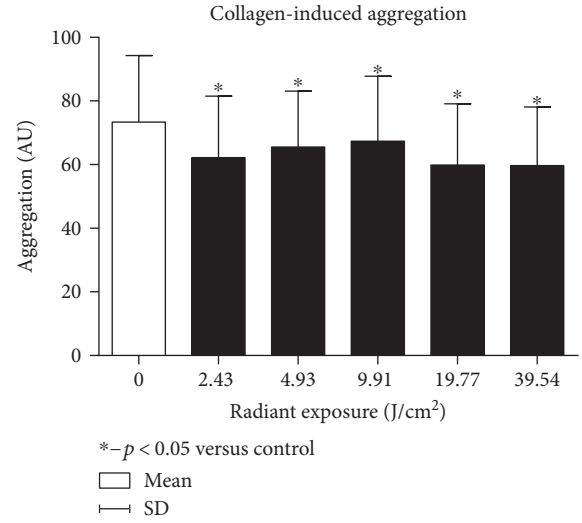

Figure 4: Dose-response effect in the platelet collagen-induced aggregation.

4. Discussion

This is, best to our knowledge, the first human study to investigate the whole blood platelet aggregation following the low-energy laser irradiation in the strictly controlled and reproducible in vitro model. In several previously conducted studies using laser radiation, it is difficult to define clearly the radiation parameters to which the blood had been subjected [4–8]. Most authors suggest that the radiation that emits a laser source reaches directly blood samples without any loss. However, the model has certain limitations

Table 1: Comparison of platelet aggregation, nitric oxide bioavailability markers, and platelet activation markers between groups.

	Laser-treated group (after LLLT) $N = 41$ (19.8 J/cm^2)	Control group (without LLLT) $N = 41$ (0 J/cm^2)	p value
ADP aggregation [AU]	66.8 ± 22.2	72.9 ± 22.7	$p = 0.0004$
TRAP aggregation [AU]	91.5 ± 21.9	105.0 ± 23.5	$p < 0.0001$
Collagen aggregation [AU]	57.7 ± 19.6	64.7 ± 22.3	$p = 0.0001$
L-arginine [μmol/l]	37.6 ± 10.4	38.1 ± 11.5	ns
ADMA [μmol/l]	0.40 ± 0.07	0.42 ± 0.06	ns
SDMA [μmol/l]	0.23 ± 0.08	0.24 ± 0.07	ns
sP-selectin [ng/ml]	26.9 ± 9.8	25.58 ± 9.8	ns
PF4 [ng/ml]	541.9 ± 521.5	519.6 ± 501.2	ns

TRAP: thrombin receptor activating peptide; ADMA: asymmetric dimethylarginine; SDMA: symmetric dimethylarginine; PF4: platelet factor 4.

especially when semiconductor laser is used, which has already been proved by Hadis et al. [12]. Such simplification makes the results of individual experiments incomparable. In order to avoid the bias, measurements of laser radiation reaching the blood samples were performed. We are aware that the accuracy of measurements may slightly differ from the actual radiation dose. This phenomenon should be related to the difference in radius (10 mm versus 20 mm), the measuring system's manual meter PMD100D and the plastic wells used to store the blood during exposure. However, taking into account the diameter of the scattered laser beam (20 mm), the central position of the blood samples during irradiation, <1 mm thin blood layer, this is the first attempt to describe precisely the experimental model to make it reproducible in subsequent studies. An effective irradiation dose can be assumed to adhere to that one measured by us. The radiation values were different than these ones expected, which had been determined on the basis of theoretical calculations. Due to differences in the radiation doses of LLLT used in studies conducted so far [4–8] and to the absence of evidence for the use of one particular dose, we were the first to perform the initial part of the experiment using such wide-range radiation doses, which subsequently allowed us to select the most effective radiation dose and to illustrate the dose-response effect.

Since the low-energy laser radiation is used in everyday clinical practice [2, 13, 14], we decided to test the whole blood platelet aggregation response. Because the study group was homogeneous, we obtained the model of "physiological" blood response to the LLLT action. Selection of healthy volunteers allowed to limit the number of potential factors affecting the obtained results. In the study, we used the wavelength of $\lambda = 662$ nm. The available data suggests [15] that two wavelength ranges $\lambda = 600$–700 nm and $\lambda = 800$–900 nm are used to modify platelet function. The radiation of these wavelengths is within the "therapeutic window" for LLLT. It is characterized by smaller absorption by water molecules and promotes the action on blood components. The thermal effect of radiation does not appear when the irradiance is below 100 mW/cm^2 (in our study, the irradiance of 53 mW/cm^2 was used and the absolute increase of 1.5°C was observed in blood temperature, which recovered to the baseline values within 3 minutes). Considering the fact that the temperature during the analysis in the multiplate aggregometer reaches 37°C, we assume that the mentioned above increase of 1.5°C has a negligible effect on the observed results.

The results of aggregation have shown that low-energy laser radiation reduces platelet aggregation in response at all tested doses of energy and all three agonists used (ADP, TRAP, and collagen). Noteworthy, we are the first to demonstrate that the antiplatelet effect of low-energy laser radiation also applies to the whole blood preparations. The studies published up to date [4–7] were conducted on platelet-rich plasma (PRP). Brill et al. [7] showed reduced activity of platelet aggregation following LLLT ($\lambda = 632.8$ nm and energy of 0.4–4.2 J) when measured in a classical optical aggregometer in response to TRAP. Interestingly, this effect was dose- and time-elapsing-from-exposure-dependent. Mohan et al. [5] ($\lambda = 1060$ nm and energy 10–50 J) observed similar results—the authors showed a reduction in aggregation response to collagen, ADP, and ristocetin.

Our data stands in opposition to the study by Hoffman and Monroe [4] where it has been demonstrated that LLLT does not modify the platelet response to thrombin. The authors postulate increased strength of formed clot and increased ability to bind certain clotting factors on the platelet surface after LLLT. It should be noted that PRP and isolated plaques as well as an irradiance dose (12 J/cm^2, wavelength 650 nm) were used, which could significantly affect the results.

Interestingly, the modulation of aggregation by LLLT is linked between the various agonists. This relationship has been demonstrated between TRAP and ADP. The conducted analyses show that the modification of the whole blood platelet aggregation response to LLLT in case of TRAP was also a predictor of changes in the ADP-induced aggregation, which could result from the cross-talk between the intracellular pathways transducing the signals derived from these two agonists. Present studies and results of this experiment may suggest that modification of the aggregation response after irradiation with a low-energy laser is most likely a consequence of the changes taking place directly in platelets or are due to paracrine effect of nonmorphotic plasma components. Hence, the plasma levels of platelet activation markers before and after irradiation were analyzed. The

measurements showed no significant differences between PF4 and sP-selectin levels in both groups. Presented data seems to be incompatible to the one presented by Brill et al. [7], where reduction in P-selectin following LLLT was noted. In our study, we investigated soluble fraction of P-selectin in the plasma and Brill focuses on P-selectin fraction located on the surface of platelets. Taking this into consideration, it can be postulated that LLLT modifies the composition of the platelet membranes but does not affect the factors released to plasma.

Nitric oxide is a potent antiplatelet factor. The low-energy laser radiation modifies cytochrome function, which results in increased nitric oxide production [8, 9]. In our study, there were no significant changes in the levels of L-arginine, ADMA, and SDMA following the LLLT when compared to the baseline values. Hence, this data suggests that LLLT does not change the nitric oxide synthesis. However, taking into consideration these results, we cannot exclude that NO is responsible for the decrease in platelet aggregation. It is possible that direct increase in NO release from blood cells (e.g., from nitroso-hemoglobin (Hb-NO)) [8, 16] is responsible for modification of aggregation. However, in our study, we did not determine the level of this fraction of NO. This would require determination of nitrate or changes in the level of cGMP as a second NO messenger.

5. Conclusions

This is the first human study to demonstrate under strictly reproducible conditions that the low-energy laser irradiation decreases the whole blood platelet aggregation in an *in vitro* model on the mechanisms independent on the nitric oxide metabolism and without significant effect on the release of platelet activation markers.

Data Access

This study was registered in the EudraCT database. The EudraCT no. 2014-001609-41 has been issued for Protocol code no. POIG.01.01.02-02-001/08project19.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper. The source of funding (a research grant) did not lead to any conflict of interests regarding publication of this manuscript nor objectivity of the data presented.

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Research Article

Edaravone, a Synthetic Free Radical Scavenger, Enhances Alteplase-Mediated Thrombolysis

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The combination of alteplase, a recombinant tissue plasminogen activator, and edaravone, an antioxidant, reportedly enhances recanalization after acute ischemic stroke. We examined the influence of edaravone on the thrombolytic efficacy of alteplase by measuring thrombolysis using a newly developed microchip-based flow-chamber assay. Rat models of embolic cerebral ischemia were treated with either alteplase or alteplase-edaravone combination therapy. The combination therapy significantly reduced the infarct volume and improved neurological deficits. Human blood samples from healthy volunteers were exposed to edaravone, alteplase, or a combination of alteplase and edaravone or hydrogen peroxide. Whole blood was perfused over a collagen- and thromboplastin-coated microchip; capillary occlusion was monitored with a video microscope and flow-pressure sensor. The area under the curve (extent of thrombogenesis or thrombolysis) at 30 minutes was 69.9% lower in the edaravone-alteplase- than alteplase-treated group. The thrombolytic effect of alteplase was significantly attenuated in the presence of

hydrogen peroxide, suggesting that oxidative stress might hinder thrombolysis. D-dimers were measured to evaluate these effects in human platelet-poor plasma samples. Although hydrogen peroxide significantly decreased the elevation of D-dimers by alteplase, edaravone significantly inhibited the decrease. Edaravone enhances alteplase-mediated thrombolysis, likely by preventing oxidative stress, which inhibits fibrinolysis by alteplase in thrombi.

1. Introduction

Alteplase is the most effective and frequently used recombinant tissue plasminogen activator (tPA) for thrombolysis in patients with acute ischemic stroke (AIS) [1]. Tissue plasminogen activators are enzymes that catalyze the conversion of plasminogen to plasmin [2].

Edaravone (Radicut®; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) is a low-specificity antioxidant that scavenges various free radicals. Edaravone exhibits neurovascular protective effects against apoptosis, necrosis, edema, and inflammatory cytokines [3–9].

Several clinical trials have shown that edaravone-alteplase combination therapy is more effective than alteplase alone in patients with AIS [1, 10–13]. Kimura et al. [14] presumed that these therapeutic effects occurred because edaravone protected the endothelium from ischemic injury, which increased the endogenous tPA levels and promoted early recanalization [14–16]. However, the currently available global clinical evidence for the efficacy of edaravone is inadequate, and more basic evidence for the efficacy of this drug in combination with alteplase is needed.

In the ischemic brains of rats, edaravone prevents endothelial cell damage and blood-brain barrier disruption [17, 18]. These *in vivo* studies showed that edaravone-alteplase combination therapy was more effective than alteplase alone in rats with occlusion of the middle cerebral artery by an intraluminal nylon filament [17, 18]. However, no reports have described the establishment of a model of thromboembolic clot-induced cerebral ischemia, which would more accurately reflect clinical morbid conditions. Therefore, we used a model of thromboembolic clot-induced cerebral ischemia *in vivo*.

A recent experimental study showed that the thrombus volume was significantly lower with edaravone-alteplase combination treatment than with alteplase alone in a model of helium-neon laser-induced thrombosis of rat mesenteric microvessels [19]. Edaravone is considered to protect the endothelium, prevents new thrombus formation by enhancing the expression of endothelial nitric oxide synthase, improves nitric oxide release, and inhibits the expression of selectin [20]. An increased nitric oxide level leads to vasorelaxation, and downregulation of selectin suppresses platelet adhesion, platelet aggregation, and leukocyte adhesion [21]. Therefore, edaravone is expected to accelerate early recanalization. However, the effects of edaravone on the blood itself, not on endothelial cells, are unknown. Current *in vitro* assays of fibrinolytic reactions, such as clot-lysis tests, thromboelastography, and rotational thromboelastometry, are generally performed in the absence of blood flow; this limits their relevance to pathologic arterial thrombosis or physiological hemostasis [22, 23]. To overcome the limitations associated with animal models and static *in vitro* assays for assessing fibrinolysis, Hosokawa et al. [24] speculated that evaluating fibrin-rich platelet thrombus formation under shear flow could be a useful model for studying thrombolytic processes in the arterial circulation.

The aim of the present study was to examine the mechanism by which edaravone promotes alteplase-mediated thrombolysis *in vitro* in human blood donated by healthy volunteers. We used the newly developed Total Thrombus-formation Analysis System (T-TAS®; Fujimori Kogyo Co., Ltd., Tokyo, Japan) to quantify thrombolysis in blood exposed to edaravone, alteplase, or alteplase and edaravone. We also examined the influence of hydrogen peroxide (H_2O_2) on alteplase-induced thrombolysis. Finally, we measured the concentration of D-dimers, which are fibrin degradation products, in platelet-rich plasma (PRP) sump solutions collected after the T-TAS assay to determine whether edaravone-alteplase combination therapy inhibits thrombogenesis or promotes thrombolysis.

2. Materials and Methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Kagoshima University (Kagoshima, Japan). The study protocol was approved by the local ethics committee of Kagoshima University, and written informed consent was obtained from all individuals prior to their participation.

2.1. Rat Model of Thromboembolic Ischemia. Thromboembolic ischemia of the middle and posterior cerebral arteries was induced in 8-week-old male Sprague–Dawley rats weighing 290 to 310 g as previously described [25], with some modifications. Anesthesia was induced and maintained with 2.5% to 3.0% isoflurane inhalation. After establishment of anesthesia, the rats were placed on the operating table in the supine position. The rectal temperature was kept at $37^{\circ}C \pm 1^{\circ}C$ from the start of anesthesia until awakening. Initially, we performed a left femoral 1 cm skin incision, inserted a 0.7×1.9 mm 24-gauge catheter (Angiocath®; Becton Dickinson Co., Fukushima, Japan) into the left femoral artery under a microscope, and withdrew 0.15 ml of arterial blood. This blood was injected into a 1.5 ml microtube containing 10 units of thrombin (Sawai Pharmaceutical, Osaka, Japan) in 50 μl of saline and kept at room temperature for 30 min. Centrifugation at 2800 rpm was performed for 2 min, and the supernatant serum was discarded. The clot was suctioned into 4-French polyvinyl chloride tubes (Atom Extension Tube; Atom Medical, Tokyo, Japan) with a 1ml disposable syringe (Termosyringe®; Terumo Co., Tokyo, Japan). Under an operating microscope, the left common, external, and internal carotid arteries were exposed through a midline incision. The external carotid artery was ligated, coagulated, and cut down just proximal to the lingual and maxillary artery

branches. All other branches of the external carotid artery were coagulated and transected. The internal carotid artery was then isolated to avoid damage to the vagus nerve. The pterygopalatine artery was ligated at its origin. The internal and common carotid arteries were clamped with small aneurysm clips. A 24-gauge catheter (SURFLO Flash®; Terumo Co.) combined with a 1 ml syringe was inserted into the internal carotid artery via a small incision in the external carotid artery stump. The clot was pushed into the internal carotid artery via a 24-gauge catheter by means of a straightened 1 mm diameter paper clip. The thrombus occluded the distal internal carotid artery, the proximal portion of the anterior cerebral artery, the middle cerebral artery, and the posterior cerebral arteries. To evaluate the infarction volume, neuromotor function, and hemorrhagic transformation at 24 h, the 5 mm clot (volume of 3.6 mm^3) was pushed into the internal carotid artery ($n = 8$ per group). After withdrawing the catheter, the external carotid artery was ligated. The temporary clip was withdrawn, and the internal carotid artery blood flow recovered.

We did not use a cerebral blood flow (CBF) monitor. Shimamura et al. [25] reported that a CBF monitor is not indispensable for this model because other surgical manipulations can be performed to establish whether brain injury and/or changes in intracranial pressure have been avoided. Additionally, dissection of the temporal muscle causes masticatory dysfunction, leading to inadequate nutrition. Instead, we used only rats with a neurological score of 3 or 4 after awakening. The rats were evaluated for neurological deficits after awakening and at 24 h after thromboembolism. A neurological grading system with a 5-point motor function scale (0–4) was used as previously described [26]. The scale was as follows: 0 = no apparent deficits, 1 = right forelimb flexion, 2 = decreased right forelimb grip when tail is pulled, 3 = spontaneous movement in all directions with right circling only when tail is pulled, and 4 = spontaneous right circling.

2.2. Drug Treatment. Three groups of rats were studied as follows: the vehicle-injected control group, alteplase group, and edaravone-alteplase group. Edaravone and alteplase were provided by Mitsubishi Tanabe Pharma Corporation. Immediately after thromboembolism, 6 mg/kg of edaravone was administered over a 20 min period via a jugular vein catheter using an infusion pump. At 20 min after thromboembolism, 3 mg/kg of alteplase was administered shortly after edaravone administration. The alteplase group received vehicle instead of edaravone, followed by alteplase treatment. The control group received injections of vehicle instead of edaravone and alteplase. The dose and timing of administration of edaravone and alteplase were determined via preliminary experiments to maximize the effect of edaravone [5]. Therefore, the dose of alteplase was lower than that in previous reports using 10 mg/kg [17, 18].

2.3. Measurement of Infarct Volume. After venous blood sampling, the rats were killed and their brains excised 24 h after thromboembolism as previously described [26]. Physiological saline was transcardially perfused before decapitation. The brain was carefully removed and cut into six 2 mm thick coronal sections from the frontal tip using a brain slicer. The slices were then immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride in phosphate buffered saline (pH 7.4) at 37°C for 10 min. After staining, the sections were scanned to determine the ischemic infarct volume. The infarctions were measured using Scion Image software versus Beta 4.0.3 (Scion Corp., Frederick, MD). The total infarct area (mm^3) was multiplied by the thickness of the brain sections to obtain the infarct volume. Additionally, the presence of visible hematomas or hemorrhagic transformation was recorded.

2.4. Rat Blood Samples and Laboratory Data. At 24 h after thromboembolism, the rats were deeply anesthetized via an intraperitoneal injection of 4% chloral hydrate (10 ml/kg). Blood samples were collected from the axillary vein.

Adverse drug reactions such as renal and hepatic disorders are occasionally observed during edaravone treatment in >5% of patients [27]. To evaluate adverse drug reactions including renal and hepatic disorders, the serum aspartate transaminase, serum alanine transaminase, blood urea nitrogen, and serum creatinine levels were measured by an enzymatic method using a Fuji DRI-CHEM Slide Kit (Fujifilm Medical, Tokyo, Japan).

2.5. Human Blood Samples. Blood samples from 10 healthy, fasting Japanese volunteers (mean age, 40.3 ± 12.7 years) were collected in plastic tubes containing 3.2% sodium citrate (Terumo Co.). None of the volunteers had taken antithrombotic drugs within 2 weeks of the study. One volunteer regularly took an over-the-counter fish oil supplement (docosahexaenoic acid 300 mg/day, eicosapentaenoic acid 100 mg/day). Normal ranges for the T-TAS analysis have not yet been defined, but the T-TAS findings of all volunteers' samples lay within 95% of the median of 123 healthy Japanese individuals who participated in our preliminary study (data not shown). Platelet-rich plasma was prepared by centrifugation at 800 rpm for 15 min, and platelet-poor plasma (PPP) was prepared by centrifugation at 3000 rpm for 15 min.

In the experiments using whole blood and PRP samples, we selected the final concentration of alteplase (500 IU/ml) and edaravone (3 μM) based on half the maximum concentration after administration to Japanese patients with AIS [5, 28]. In the experiments using PPP samples, we selected the final concentration of alteplase (50, 100, and 250 IU/ml) and edaravone (6 and 60 nM) based on the findings of a preliminary experiment. The final concentrations of vitamin C (6 μM), vitamin E (120 nM), and NAC (6 μM) were selected based on a preliminary experiment and previous reports and were expected to have efficacy equivalent to that of edaravone [29]. The final concentration of H_2O_2 (100 μM) was based on a previous report, recognizing that it is difficult to estimate the local concentration of reactive oxygen species (ROS) around the intravascular thrombi during AIS [5]. Vitamins C and E were obtained from Kanto Kagaku Co., Ltd. (Tokyo, Japan), NAC was obtained from Wako Pure

Chemicals (Osaka, Japan), and H_2O_2 was obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Samples from six healthy, fasting volunteers (mean age, 35.8 ± 7.6 years) were further selected for the experiments in which specimens were exposed to H_2O_2. These 6 volunteers were selected from the original cohort of 10 because they had a normal response to alteplase. Of the remaining volunteers, one was a nonresponder, two were incomplete responders, and one was an over-responder.

2.6. T-TAS. The thrombolytic effects of alteplase, edaravone, and the combination of alteplase and edaravone were compared with the controls under flow conditions using T-TAS in whole blood and PRP. To quantify thrombogenesis and thrombolysis under flow conditions, the T-TAS assay was performed as previously described [24]. Thrombogenesis and thrombolysis were observed in the microchip using a built-in light microscope. An antioxidant (edaravone, vitamin C or E, or NAC) was added to the blood samples 10 min before the addition of alteplase. Hydrogen peroxide was added immediately after the addition of alteplase. As soon as alteplase or H_2O_2 had been administered, each sample was perfused over a microchip coated with collagen and tissue thromboplastin to promote thrombosis at a flow rate of 4 μl/min, corresponding to an initial wall shear rate of 240 per second.

2.7. Measurement of D-Dimer Concentration in Sump Solutions of T-TAS. T-TAS sump solutions were prepared by diluting the analyzed pooled PRP samples at a 1 : 25 ratio in ethylenediaminetetraacetic acid followed by centrifugation at 800 rpm for 15 min. The concentration of D-dimers was also measured in the sump solution using an LPIA-NV7 instrument and RM73-752YLK solution (LSI Medience Corporation, Tokyo, Japan).

2.8. Fibrinolysis Assays. Fibrinolysis assays were performed to evaluate whether edaravone enhances thrombolysis by alteplase. Microplate-based fibrinolysis assays were performed at 37°C in flat-bottomed 96-well polystyrene plates (Corning; Sigma-Aldrich Japan) by monitoring turbidity changes (A_{405}) using a VersaMax microplate reader (Molecular Devices Japan, Tokyo, Japan). Calcium ions accelerate the formation of a fibrin clot from fibrinogen in the presence of thrombin. One-hundred microliter aliquots of 30% human PPP pooled from all 10 volunteers and TBSTC (8 mM Tris at pH 7.4, 0.008% Tween-20, and 12 mM calcium chloride) were prepared in the presence or absence of alteplase (0, 50, 100, or 250 IU/ml) or edaravone (0, 6, or 60 nM; $n = 5$). The concentrations of edaravone had been determined in a preliminary experiment. Edaravone was added to the samples 10 min before alteplase.

2.9. Measurement of D-Dimer Concentration in Human PPP Samples. D-dimers were measured to determine whether edaravone attenuates the inhibition of alteplase-induced fibrinolysis by H_2O_2. Four-hundred microliter aliquots of 30% human PPP pooled from all 10 volunteers and TBSTC were prepared. After incubation at 37°C for 15 min, H_2O_2 (100 μM), edaravone (60 nM), or vehicle was added. After incubation at 37°C for 10 min, alteplase (100 IU/ml), plasmin (250 μg/ml), or vehicle was added. After fibrin deposition had occurred by incubating at 37°C for 10 min, aprotinin (400KIU/ml) was added. After centrifugation at maximum speed for 5 min, the concentration of D-dimers was measured using an ACL TOP automated analyzer (Instrumentation Laboratory, Bedford, MA) ($n = 5$).

2.10. Statistical Analysis. The neurological score, infarction volume, hemorrhagic transformation rate, and blood test data were analyzed using the Steel–Dwass method or Bonferroni–Dunn method as appropriate in multiple comparisons.

The area under the curve at 30 min (AUC30) was calculated to evaluate the extent of thrombogenesis or thrombolysis. The AUC30 represents the area under the flow-pressure curve (<80 kPa) 30 min after the start of assay, as previously described [24]. The AUC30 was also used to quantify the impairment of thrombus formation when occlusion is not achieved during an assay. Comparison between two groups was performed using the paired t-test or Wilcoxon signed-rank test as appropriate.

Absorbance data were analyzed by repeated-measures two-way analysis of variance followed by Bonferroni's test.

Data are presented as mean ± standard deviation unless otherwise indicated. Differences with $P < 0.05$ were considered statistically significant. All analyses were performed using SPSS Statistics (version 20; IBM Corp., Armonk, NY).

3. Results

3.1. Edaravone-Alteplase Combination Reduces Infarct Volume and Improves Neuromotor Function in Rats. We evaluated our rat thromboembolic stroke model, in which the distal internal carotid artery, proximal portion of the anterior cerebral artery, middle cerebral artery, and posterior cerebral arteries were occluded by autologous thrombi (Figure 1(b)). We compared the vehicle-injected control group, alteplase group, and edaravone-alteplase combination group, which allowed us to investigate the therapeutic effects of edaravone-alteplase combination therapy (Figure 1(a)).

We did not use a CBF monitor according to a previous report [25]. The neurological score of all rats after awakening was 3 or 4, and the differences among the three groups of rats that were assigned to the different treatments were not statistically significant (Table 1). Therefore, we were able to induce cerebral ischemia without using a CBF monitor.

We then evaluated the infarct volume, neuromotor function, hemorrhagic transformation, and adverse drug reactions at 24 h after ischemia in the three groups. Compared with the controls, alteplase significantly reduced the infarct volume ($P < 0.05$) (Figures 1(b) and (c)). However, the infarct volume was further significantly decreased in rats receiving edaravone-alteplase ($P < 0.05$) (Figures 1(b) and (c)). The neurologic score in rats receiving alteplase was significantly better than that in the controls ($P < 0.05$) (Figure 1(d)). Additionally, the rats treated with edaravone-alteplase showed significantly better neurologic scores than the rats treated with alteplase ($P < 0.01$) (Figure 1(d)). Meanwhile, the rate of hemorrhagic transformation tended to be lower

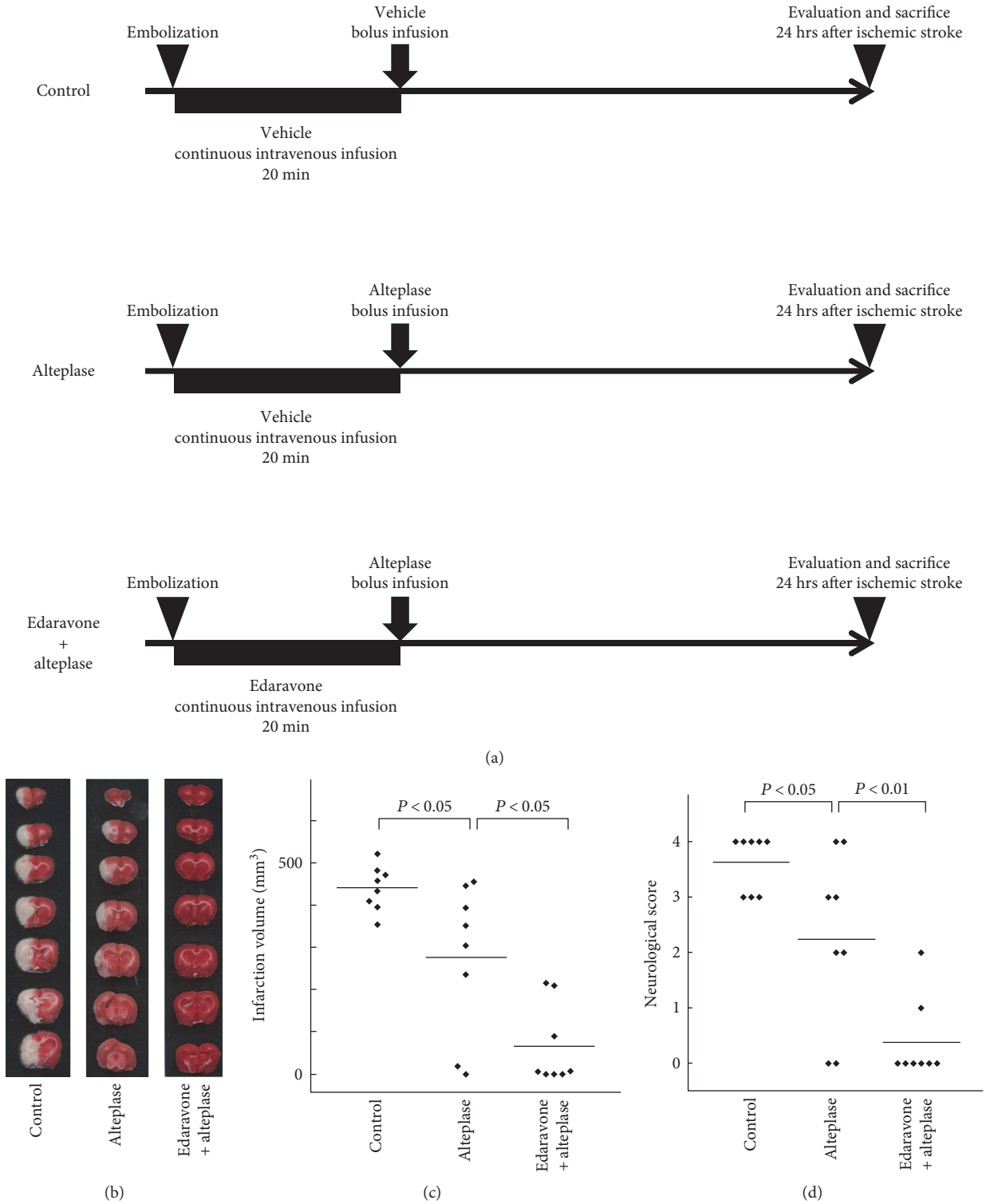

Figure 1: Effect of edaravone-alteplase combination therapy in a rat model of thromboembolic clot-induced cerebral ischemia. (a) The experimental groups included the vehicle-injected control group, alteplase group, and edaravone-alteplase combination group ($n = 8$ per group). Rats were killed 24 hours after establishment of cerebral ischemia. (b) Representative figures of 2,3,5-triphenyltetrazolium chloride-stained brain sections of rats. Normal brain tissue stains deep red, and ischemic lesions are white (unstained). (c) Infarct volume in the control, alteplase, and edaravone-alteplase groups. (d) Neurological score in the control, alteplase, and edaravone-alteplase groups. The horizontal lines represent the mean values.

Table 1: Therapeutic Effects and Laboratory Data in the Rat Thromboembolic Ischemic Model.

	Control	Alteplase	Edaravone-alteplase combination
	n	*n*	*n*
Total number of rats			
	8	8	8
Neurological score after awakening			
3	5	4	3
4	3	4	5
Hemorrhagic transformation			
	0	2	0
Blood test data			
AST (U/L)	233.0 ± 241.5	182.5 ± 30.0	234.4 ± 125.1
ALT (U/L)	34.0 ± 11.8	34.4 ± 3.9	42.3 ± 19.2
BUN (mg/dl)	12.4 ± 1.3	13.2 ± 1.4	13.4 ± 1.9
Cr (mg/dl)	0.18 ± 0.07	0.18 ± 0.05	0.19 ± 0.07

Blood test data are shown as mean ± standard deviation. BUN: blood urea nitrogen; Cr: creatinine; AST: aspartate transaminase; ALT: alanine transaminase.

Table 2: Erythrocyte, leukocyte, and platelet counts in whole blood and platelet-rich plasma used in the Total Thrombus-formation Analysis System assay.

	Whole blood	Platelet-rich plasma
Erythrocyte count ($\times 10^6/\mu$l)	4.78 ± 0.42	0.01 ± 0.01
Leukocyte count ($\times 10^3/\mu$l)	5.78 ± 1.07	0.02 ± 0.01
Platelet count ($\times 10^3/\mu$l)	224 ± 4	313 ± 105

Values are shown as mean ± standard deviation.

in the edaravone-alteplase group than in the alteplase group. However, the differences were not statistically significant among the three groups (Table 1).

Adverse drug reactions, including renal and hepatic disorders, were not apparent because the serum aspartate transaminase, serum alanine transaminase, blood urea nitrogen, and serum creatinine levels were not significantly different among the three groups (Table 1).

In conclusion, edaravone synergized with acute alteplase treatment in this experimental thrombotic stroke model.

3.2. Characteristics of Blood Samples Obtained from Healthy Volunteers. The mean erythrocyte, leukocyte, and platelet counts of whole blood and PRP samples are shown in Table 2. These lay within the normal ranges for healthy Japanese individuals.

3.3. Edaravone Enhances Alteplase-Mediated Thrombolysis in Human Whole Blood. To clarify the mechanism of the synergistic effects of edaravone in the animal model (Figure 1), we next evaluated whether edaravone enhances alteplase-mediated thrombolysis under flow conditions using the T-TAS in human whole blood (Figure 2). After perfusion had started, plentiful small white thrombi were observed adhering to the coated surface. The thrombi gradually increased in size and merged with each other, leading to capillary occlusion in 9 to 10 min in the control and edaravone groups (data not shown). In the alteplase and edaravone-alteplase groups, capillary occlusion occurred at 18 to 19 min. However, in the edaravone-alteplase group, the thrombi had dissolved within 27 to 28 min (Figure 2(a)). Treatment with alteplase alone had a limited effect on thrombus firmness, but in the presence of edaravone, thrombus firmness diminished as evidenced by the frequent collapse of thrombi.

Thrombus formation caused microcapillary occlusion in all control samples not exposed to alteplase, and thrombolysis was evident in the majority of samples exposed to alteplase. However, the perfused capillary became completely occluded in three samples exposed to alteplase (one in which there was no response to alteplase [10%] and two in which there was an incomplete response [20%]). An exaggerated response to alteplase was observed in one sample (10%). In alteplase nonresponders, thrombolysis as evidenced by reduced thrombus firmness and the frequent collapse of thrombi was observed in samples to which both edaravone and alteplase had been added. The sample provided by the volunteer who regularly took the fish oil supplement exhibited a normal response to alteplase.

Microcapillary occlusion occurred in all control samples and the edaravone group and in three samples in the alteplase group but in none of the samples in the edaravone-alteplase group (Figure 2(b)). We observed a characteristic periodic flow-pressure pattern that reflected the collapse of thrombi, consistent with the lack of microcapillary occlusion in the edaravone-alteplase group. The synergistic effect of edaravone-alteplase combination therapy was evaluated by calculating the AUC30, which was significantly lower in the edaravone-alteplase group than in the alteplase group ($P < 0.01$) (Figure 2(c)). There was no significant difference in the AUC30 between the control group (1574.9 ± 108.8) and edaravone group (1495.2 ± 153.7) ($P = 0.997$). In conclusion, edaravone enhanced alteplase-mediated thrombolysis in human whole blood.

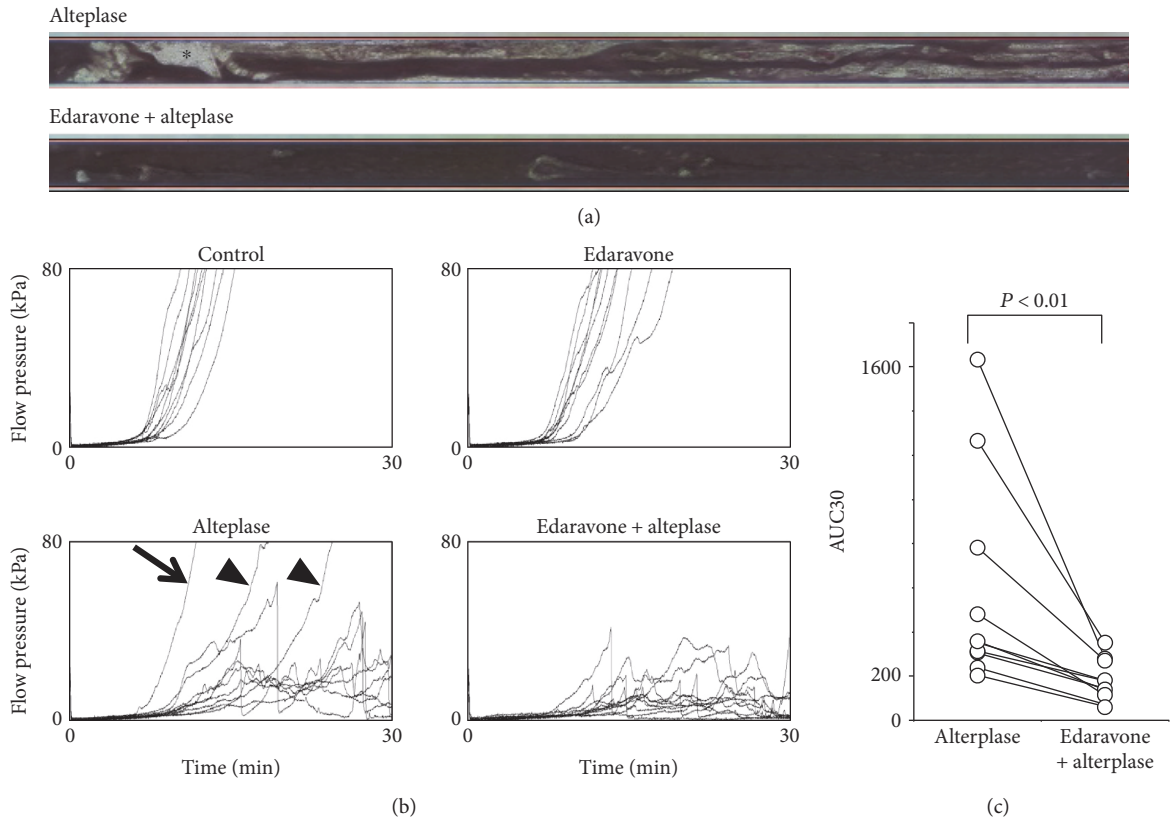

Figure 2: Effect of edaravone on alteplase-induced thrombolysis under flow conditions in whole blood. (a) Typical still videomicroscopy images of thrombogenesis and thrombolysis over 27 to 28 min in samples exposed to alteplase or edaravone-alteplase. The asterisk (white area) indicates thrombi. (b) Flow-pressure curves in the control, edaravone, alteplase, and edaravone-alteplase groups ($n = 10$). The arrow indicates an alteplase nonresponder, and the arrowhead indicates incomplete responders. (c) AUC30 in the alteplase and edaravone-alteplase groups. AUC30: area under the curve at 30 min.

3.4. Other Antioxidants Enhance Alteplase-Mediated Thrombolysis in Human Whole Blood. Thrombolysis enhancement may be an edaravone-specific effect in human whole blood (Figure 2). Whether antioxidants other than edaravone also have a thrombolysis-enhancing effect is unknown. We examined and compared the synergistic effect of alteplase with other general antioxidants under flow conditions using the T-TAS in whole blood (Figure 3). Microcapillary occlusion occurred in two of the samples in the alteplase group but in none of the samples in which vitamin E was coadministered with alteplase (Figure 3(a)). In the alteplase-vitamin E group, the AUC30 was significantly lower than that in the alteplase alone group ($P < 0.05$) (Figure 3(b)). The AUC30 was also significantly lower in the alteplase-NAC group (316.8 ± 246.1) than the alteplase alone group (601.6 ± 457.4) ($P < 0.05$). However, there was no significant difference in the AUC30 between the alteplase-vitamin C group (469.0 ± 500.1) and the alteplase alone group ($P = 0.334$). These findings confirm that some general antioxidants have a thrombolysis-enhancing effect similar to that of edaravone.

3.5. Reactive Oxygen Species Inhibit Alteplase-Mediated Thrombolysis in Human Whole Blood. Edaravone and other antioxidants (vitamin E and NAC) might enhance alteplase-mediated thrombolysis in whole blood (Figure 3). ROS may be the key molecular targets of these antioxidants, including edaravone. We examined whether ROS such as H_2O_2 affect thrombogenesis or thrombolysis in whole blood under flow conditions using the T-TAS (Figure 4). Addition of 100 μM H_2O_2 to samples exposed to alteplase resulted in microcapillary occlusion in a greater proportion of samples than those exposed to alteplase alone (Figure 4(a)). The AUC30 in the H_2O_2 alone group was broadly comparable with that in controls ($P = 0.787$) (Figure 4(b)), but the AUC30 was significantly higher in the alteplase-H_2O_2 group than in the alteplase alone group ($P < 0.05$) (Figure 4(c)). Therefore, ROS inhibited thrombolysis but did not induce thrombogenesis at this concentration (100 μM H_2O_2).

3.6. Edaravone Enhances Alteplase-Mediated Thrombolysis in Human Plasma Components. Pertaining to which components of whole blood are affected by edaravone is unclear. Comparison of whole blood and PRP samples revealed negligible numbers of erythrocytes and leukocytes in PRP samples (Table 2). Therefore, which blood components are affected by edaravone may become evident by comparing its effect between whole blood and PRP samples. Therefore, we examined the thrombolytic effects of alteplase, edaravone, and their combination under flow conditions using the

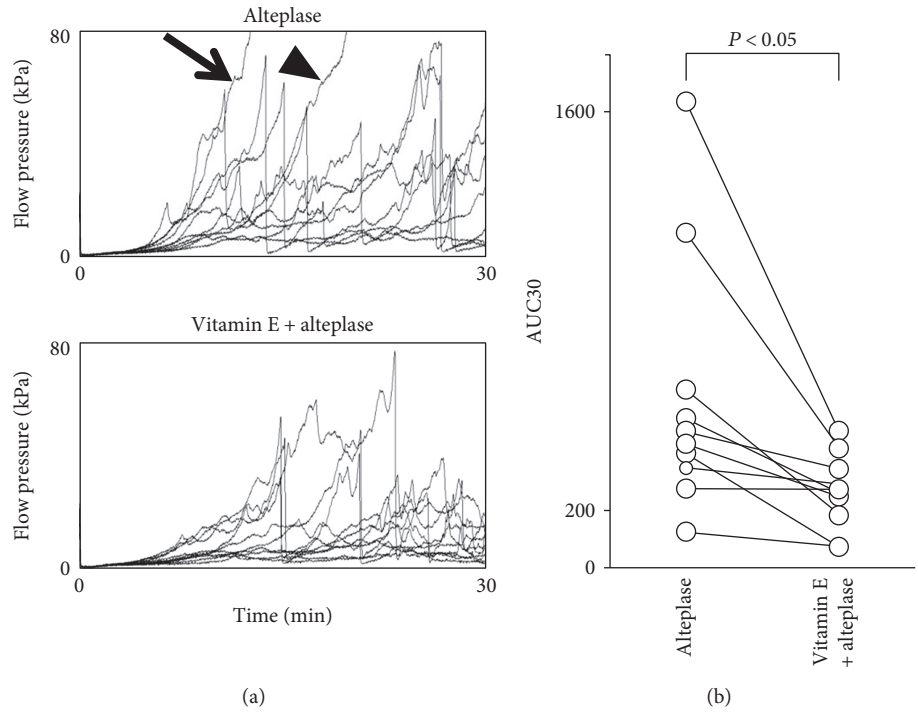

Figure 3: Effect of vitamin E on alteplase-induced thrombolysis under flow conditions in whole blood. (a) Flow-pressure curves in the alteplase and vitamin E-alteplase groups ($n = 10$). The arrow indicates an alteplase nonresponder, and the arrowhead indicates an incomplete responder. (b) AUC30 in the alteplase and vitamin E-alteplase groups. AUC30: area under the curve at 30 min.

T-TAS in PRP (Figures 5(a) and (b)). Exposure of PRP (Table 2) to alteplase, edaravone, or edaravone-alteplase under flow conditions reduced the proportion in which microcapillary occlusion occurred (Figure 5(a)). The synergistic effect of alteplase and edaravone was reflected by a significantly lower AUC30 in the edaravone-alteplase than alteplase group ($P < 0.05$) (Figure 5(b)). Therefore, the thrombolysis-enhancing effect of edaravone was confirmed in PRP samples.

The thrombolysis-enhancing effect of edaravone was confirmed objectively and quantitatively in whole blood and PRP (Figures 2, 5a, and 5b). However, whether a low AUC on the T-TAS inhibits thrombogenesis or enhances thrombolysis remains unclear. In one ex vivo study, alteplase dissolved retrieved human cerebral thromboemboli and induced D-dimers (i.e., fibrin degradation products) and minimum protein fragment [30]. No studies have determined whether edaravone enhances induction of D-dimer release by alteplase. Therefore, we evaluated D-dimers in PRP sump solutions after measuring PRP samples using the T-TAS to confirm the thrombolysis-enhancing effect of edaravone in healthy human blood samples (Figure 5(c)). The mean concentration of D-dimers in the PRP sump solutions collected after the T-TAS assay was significantly higher in the edaravone-alteplase group (10.2 ± 3.2 mg/ml) than in the alteplase alone group (7.3 ± 1.6 mg/ml) ($P < 0.05$) (Figure 5(c)), demonstrating an inverse relationship between the AUC30 measured by the T-TAS and the D-dimer concentration. The D-dimer concentrations in all control samples (100%) were <0.03 mg/ml, which is below the measurable range. In the edaravone alone group, the D-dimer concentration was <0.03 mg/ml in 8 of 10 samples (80.0%) and 0.03 and 1.51 mg/ml in the remaining samples. These findings suggest that thrombolysis as evaluated by the T-TAS is strongly correlated with the D-dimer level.

The synergistic effect of edaravone-alteplase combination therapy was confirmed objectively by the AUC30 and quantitatively in both whole blood and PRP (Figures 2 and 5). However, the effects of edaravone-alteplase combination therapy on either platelets or plasma are unclear because both platelets and plasma are present in both whole blood and PRP samples. However, almost no platelets are present in PPP samples. Therefore, pertaining to which blood components are affected by edaravone may be clarified by comparing its effect separately in whole blood samples, PRP samples, and PPP samples. PPP samples could not be evaluated because the capillaries did not become occluded in the T-TAS assay. The lack of platelets in PPP meant that the microcapillaries did not become occluded. Therefore, we examined the effects of alteplase, edaravone, and their combination on fibrinolysis in PPP using a method other than the T-TAS (Figure 6). In the alternative turbidity assay, exposure of PPP to alteplase (50, 100, or 250 IU/ml) caused dose-dependent fibrinolysis (Figure 6(a)). In PPP samples exposed to alteplase at 100 IU/ml, fibrinolysis was significantly enhanced by addition of edaravone at 60 nM ($P < 0.001$) (Figure 6(b)), but not by edaravone at 6 nM ($P = 0.140$). Therefore, the thrombolysis-enhancing effect of edaravone was confirmed in PPP samples.

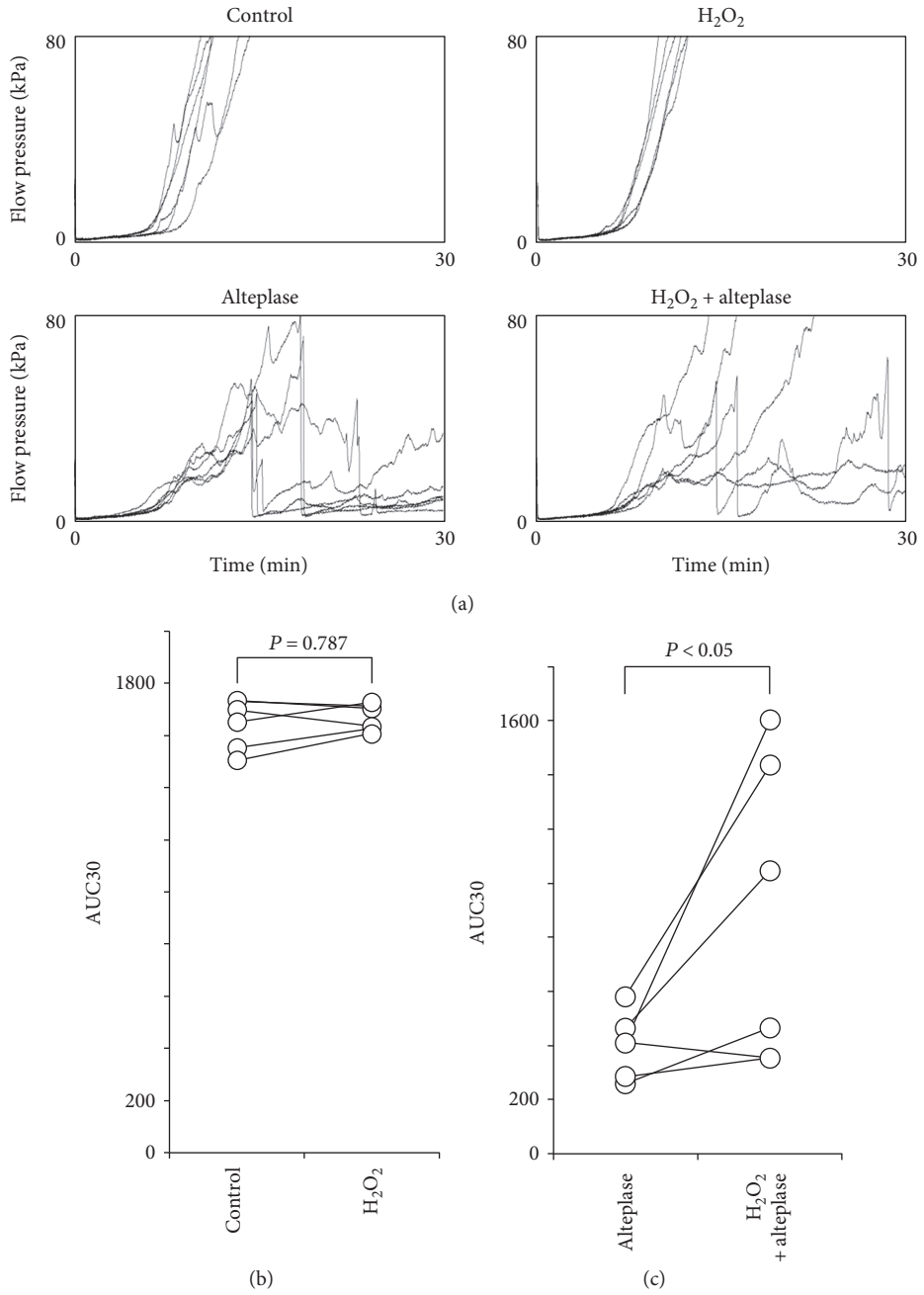

Figure 4: Effect of H_2O_2 on alteplase-induced thrombolysis under flow conditions in whole blood. (a) Flow-pressure curves in the control, H_2O_2, alteplase, and H_2O_2-alteplase groups ($n = 6$). (b) AUC30 in the control and H_2O_2 groups. (c) AUC30 in the alteplase and H_2O_2-alteplase groups. AUC30: area under the curve at 30 min; H_2O_2: hydrogen peroxide.

The D-dimer concentration was significantly higher in PPP samples exposed to alteplase at 100 IU/ml than in controls ($P < 0.05$) (Figure 6(c)). Moreover, in samples exposed to alteplase at 100 IU/ml, the D-dimer concentration was significantly reduced by the addition of H_2O_2 at 100 μM ($P < 0.05$) (Figure 6(c)). Nevertheless, in samples exposed to alteplase at 100 IU/ml and H_2O_2 at 100 μM, the D-dimer concentration was significantly increased by the addition of edaravone at 60 nM ($P < 0.05$) (Figure 6(c)). These observations confirm the mechanism of the thrombolysis-enhancing effect of edaravone. ROS may inhibit alteplase-mediated thrombolysis, which is prevented by edaravone. Meanwhile, alteplase but not plasmin was enhanced by edaravone. In PPP samples exposed to plasmin (3.35 ± 2.08 μg/ml) compared with plasmin and edaravone (2.03 ± 2.23 μg/ml), the D-dimer concentration was not increased by the addition of edaravone.

4. Discussion

The synergistic effects of edaravone-alteplase combination therapy have long been considered due to the neurovascular

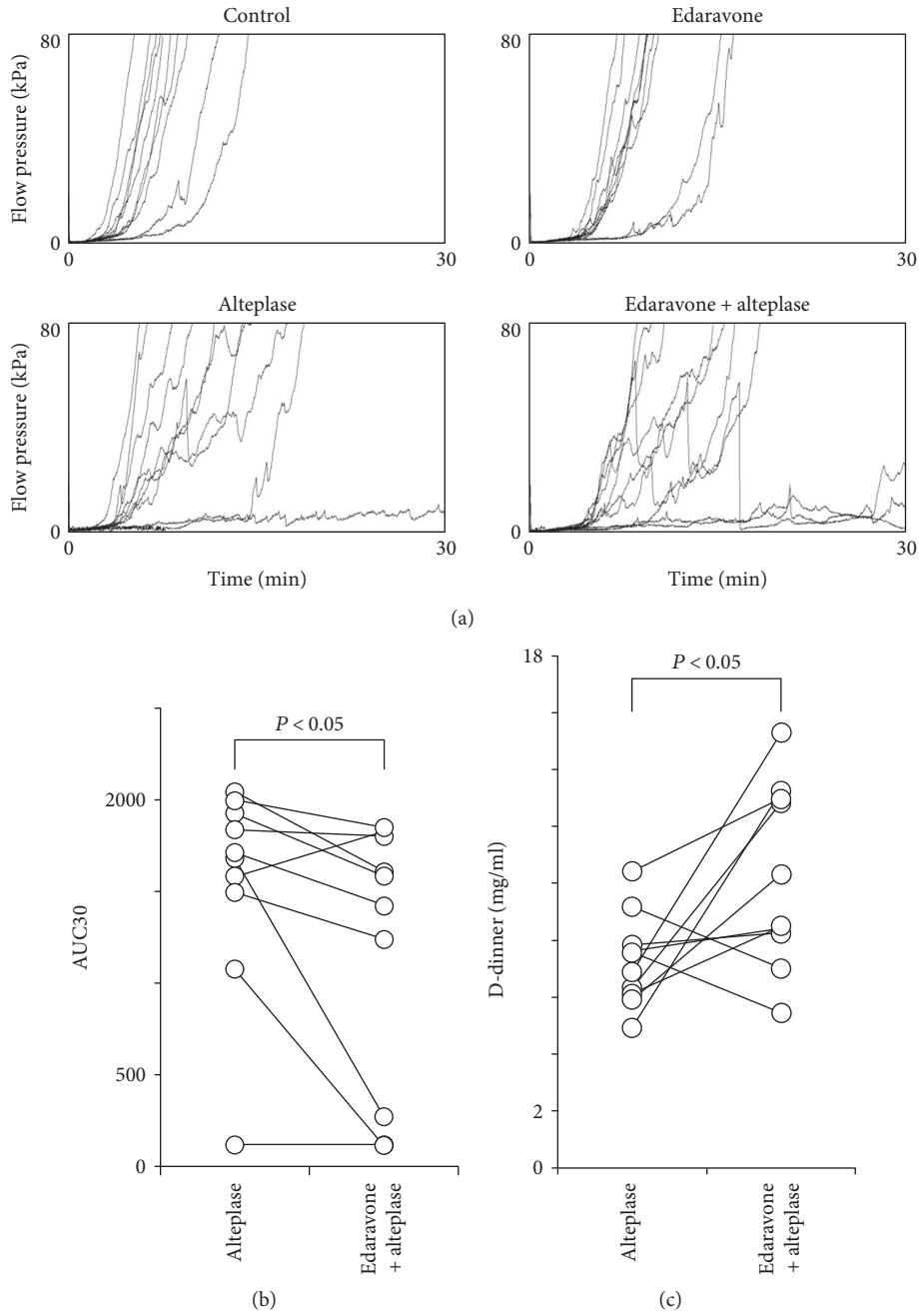

Figure 5: Effect of edaravone on alteplase-induced thrombolysis in PRP. (a) Flow-pressure curves in the control, edaravone, alteplase, and edaravone-alteplase groups in PRP under flow conditions ($n = 10$). (b) AUC30 in the alteplase and edaravone-alteplase groups in PRP. (c) D-dimer concentration in sump solutions after measurement using the Total Thrombus-formation Analysis System in PRP in the alteplase and edaravone-alteplase groups ($n = 10$). AUC30: area under the curve at 30 min; PRP: platelet-rich plasma.

protective effect of edaravone targeting ROS and matrix metalloproteinase-9 [1, 17, 18]. However, we found that, although edaravone enhanced alteplase-mediated thrombolysis, edaravone alone neither attenuated thrombogenesis nor enhanced thrombolysis. This suggests that edaravone suppresses the inhibitory action of ROS on alteplase-mediated thrombolysis (Figure 7). Our results are in agreement with previous clinical studies [10–13].

The enhancing effect of edaravone against alteplase-mediated thrombolysis was confirmed in PRP, PPP, and whole blood. Whole blood contains leukocytes, unlike PRP or PPP. Moreover, H_2O_2 inhibited alteplase-mediated thrombolysis in both whole blood samples and PPP. Edaravone may enhance alteplase-mediated thrombolysis by inhibiting generation of ROS by platelets, plasma components, and leukocytes. Platelets are also reported to produce ROS and to release platelet-derived exosomes that in turn can generate ROS [31]. The effect of edaravone that we observed in PRP and PPP samples may reflect its activity against platelet- or exosome-derived ROS.

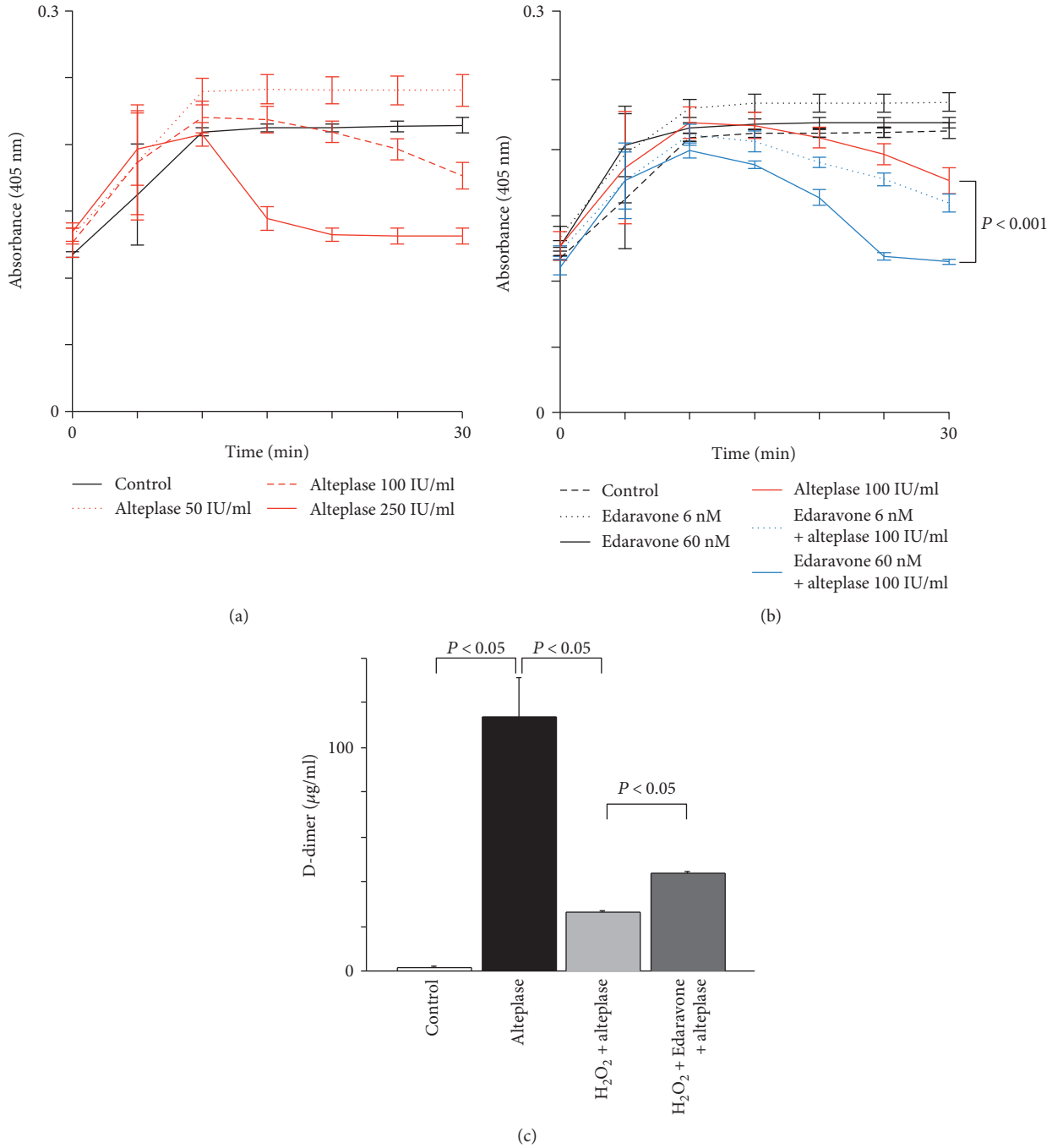

Figure 6: Effect of edaravone on alteplase-induced fibrinolysis in PPP. (a) Effects of alteplase (50, 100, or 250 IU/ml) in pooled platelet-poor plasma ($n = 5$). Error bars represent standard deviation. (b) Effects of edaravone (6 or 60 nM), alteplase (100 IU/ml), or their combination on alteplase-induced thrombolysis in pooled PPP ($n = 5$). Error bars represent standard deviation. (c) Measurement of D-dimer concentration to evaluate effects of edaravone (60 nM) on H_2O_2 (100 μM)-inhibited fibrinolysis by alteplase (100 IU/ml) in pooled PPP ($n = 5$). Error bars represent standard deviation. PPP: platelet-poor plasma; H_2O_2: hydrogen peroxide.

Although we were unable to identify the precise mechanism by which edaravone enhances the activity of alteplase, previous *in vitro* and *in vivo* studies revealed that tPA induces ROS and that ROS inhibit thrombolysis. Tissue plasminogen activators are Mac-1 (CD11b/CD18) ligands; Mac-1 mediates adhesion-dependent H_2O_2 production by human neutrophils [32]. Moreover, edaravone appears to ameliorate alteplase-induced oxidative stress in the rat brain, such as 4-hydroxy-2-nonenal and N-(hexanoyl)-lysine (lipid peroxidation markers), 8-hydroxy-2′-deoxyguanosine (a DNA oxidation marker), and advanced glycation end products (protein oxidation markers) [28]. Furthermore, ROS may induce plasminogen activator inhibitor-1 (PAI-1), while antioxidants abolish the induction of PAI-1 [33]. Therefore, the presence of an antioxidant, such as edaravone, may enhance thrombolysis by a pleiotropic mechanism.

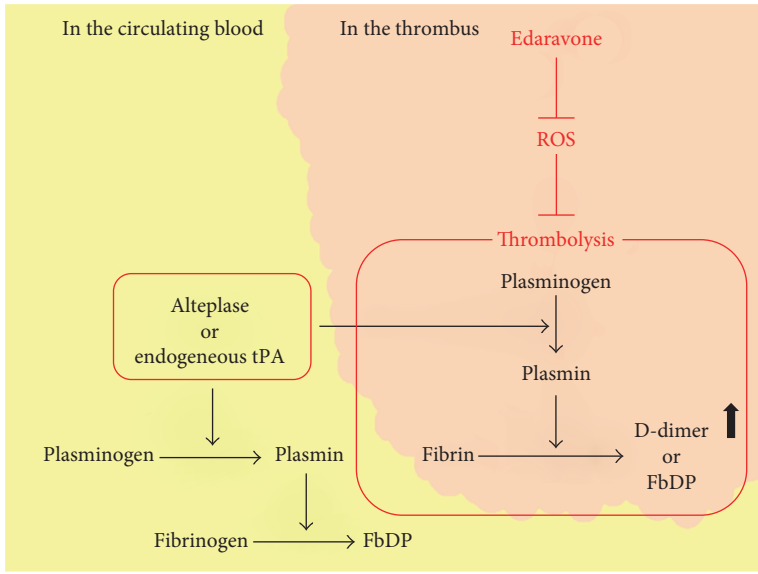

Figure 7: Schema showing the potential actions of edaravone against reactive oxygen species in thrombolytic pathways. Those in red indicate mechanisms suggested by the findings of our study. tPA: tissue plasminogen activator; FbP: fibrin degradation product; FgDP: fibrinogen degradation product.

Edaravone enhances the thrombolytic effect of alteplase; thus, edaravone-alteplase combination therapy may increase the incidence of adverse events such as intracerebral hemorrhage in patients treated for AIS. We found that edaravone had no thrombolytic effect when administered alone in coagulating blood. Edaravone may inhibit hemorrhagic transformation. This may be explained by a reduction in the frequency of adverse events caused by alteplase: the rate of development of symptomatic intracerebral hemorrhage attributed to alteplase infusion is reportedly negatively correlated with the rate of combined treatment with edaravone according to several clinical trials. [1]. This was also evident in our study. We found that alteplase but not plasmin was enhanced by edaravone. The fibrin-binding affinity of alteplase can be impaired by exposure to ROS, and the characteristic advantage of thrombus selectivity of alteplase in both spontaneous thrombolysis and thrombolytic therapy may be diminished in environments where ROS are plentiful [34]. Plasminogen exists in both circulating blood and thrombi, and plasmin degrades both fibrinogen and fibrin. Therefore, alteplase may activate plasminogen in the circulating blood rather than in thrombi under ROS-rich conditions. This would result in the production of FDP from fibrinogen in the circulating blood, while the fibrin in thrombi may decompose to a lesser degree. Edaravone-alteplase combination therapy may increase the affinity of alteplase for fibrin and cause plasmin activation to be selective for thrombi. Therefore, fibrinogen degradation associated with plasminogen activation in the circulating blood, which is not in thrombi, is less likely to occur. This may reduce the risk of bleeding tendencies. Our results are in agreement with previous clinical studies [1]. Edaravone may enhance thrombolysis and inhibit adverse events such as intracerebral hemorrhage because edaravone enhances alteplase-mediated thrombolysis, likely by preventing inhibition of alteplase-induced fibrinolysis by oxidative stress in thrombi (Figure 7).

Edaravone is not currently licensed for use in Western countries. A new formulation and dosing regimen was recently evaluated in Finland, the Netherlands, and the United Kingdom, which may promote the use of edaravone more widely throughout the European Union [35]. Meanwhile, the US Food and Drug Administration approved edaravone for the treatment of amyotrophic lateral sclerosis in 2016 [36, 37]. Therefore, a study of edaravone-alteplase combination therapy in Western countries might also be carried out in the near future. The outcomes of future studies may show whether such combination therapy is a breakthrough treatment for AIS.

4.1. Study Limitations. In clinical practice, alteplase is administered after thrombosis and blood vessel occlusion. In the present experiment, however, it was added before thrombosis was initiated. Furthermore, the reduction in the AUC30 in the T-TAS assay that we observed does not illuminate whether thrombogenesis is being inhibited or thrombolysis is being promoted. However, the elevated D-dimer concentration that we found in the sump solutions suggests that thrombolysis (lysis of fibrin clots) occurred after thrombogenesis, implying that edaravone was enhancing alteplase-mediated thrombolysis in the T-TAS assay.

Direct determination of oxidative stress such as that caused by ROS or free radicals is not easy. Moreover, obtaining high reproducibility is difficult because the circadian variation of endogenous tPA or PAI-1 is intense, and these agents are very unstable. Meanwhile, the direct binding affinity of alteplase, H_2O_2, and edaravone against precipitated fibrin should be evaluated, but we could not perform such an evaluation on an Octet system (Pall ForteBio, Fremont, CA) by analysis method of interbiomolecule interaction.

5. Conclusions

We investigated the effect of edaravone on alteplase-induced thrombolysis using a rat model of thromboembolic clot-induced cerebral ischemia (severe cardioembolic cerebral infarction *in vivo*). Moreover, we examined the mechanism by which edaravone promotes alteplase-mediated thrombolysis *in vitro* in human blood donated by healthy volunteers using a newly developed microchip-based flow-chamber assay (the Total Thrombus-formation Analysis System) to perform a quantitative analysis under flow conditions. We showed that edaravone is an enhancer of alteplase, although previous reports have only shown the synergistic effect of edaravone as a neuroprotectant. The thrombolytic effect of alteplase was significantly attenuated in the presence of hydrogen peroxide, suggesting that oxidative stress might hinder thrombolysis. Edaravone alone did not influence thrombosis or thrombolysis. Edaravone enhances alteplase-mediated thrombolysis *in vitro*, likely by acting as an antioxidant to prevent free radical-related inhibition of alteplase activity on thrombi. Furthermore, edaravone significantly attenuated inhibition of alteplase-induced fibrinolysis by hydrogen peroxide as shown by the measurement of plasma D-dimers in human platelet-poor plasma.

Disclosure

Tomoka Nagasato, Hisayo Sameshima, and Kazuya Hosokawa are employees of Fujimori Kogyo Co., Ltd.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Kiyoshi Kikuchi conducted the study, analyzed the data, and wrote the manuscript. Ketaro Setoyama, Ko-ichi Kawahara, Tomoka Nagasato, Takuto Terashi, Koki Ueda, Kazuki Nakanishi, Shotaro Otsuka, Naoki Miura, Hisayo Sameshima, Kazuya Hosokawa, Yoichiro Harada, Binita Shrestha, Mika Yamamoto, Yoko Morimoto-Yamashita, Haruna Kikuchi, Ryoji Kiyama, Chinatsu Kamikokuryo, Salunya Tancharoen, Harutoshi Sakakima, Motohiro Morioka, Eiichiro Tanaka, and Takashi Ito all helped with data analysis. Ikuro Maruyama designed the study and analyzed the data.

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Research Article

Effect of ALDH2 on High Glucose-Induced Cardiac Fibroblast Oxidative Stress, Apoptosis, and Fibrosis

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Our study aimed firstly to observe whether ALDH2 was expressed in neonate rat cardiac fibroblasts, then to investigate the effect of activation of ALDH2 on oxidative stress, apoptosis, and fibrosis when cardiac fibroblasts were subjected to high glucose intervention. Cultured cardiac fibroblasts were randomly divided into normal (NG), NG + Alda-1, high glucose (HG), HG + Alda-1, HG + Alda-1 + daidzin, HG + daidzin, and hypertonic groups. Double-label immunofluorescence staining, RT-PCR, and Western blot revealed ALDH2 was expressed in cardiac fibroblasts. Compared with NG, ALDH2 activity and protein expression were reduced, and cardiac fibroblast proliferation, ROS releasing, 4-HNE protein expression, collagen type I and III at mRNA levels, and the apoptosis rate were increased in HG group. While in HG + Alda-1 group, with the increases of ALDH2 activity and protein expression, the cardiac fibroblast proliferation and ROS releasing were decreased, and 4-HNE protein expression, collagen type I and III at mRNA levels, and apoptosis rate were reduced compared with HG group. When treated with daidzin in HG + Alda-1 group, the protective effects were inhibited. Our findings suggested that ALDH2 is expressed in neonate rat cardiac fibroblasts; activation of ALDH2 decreases the HG-induced apoptosis and fibrosis through inhibition of oxidative stress.

1. Introduction

Diabetes mellitus (DM) is one of the most serious chronic diseases in the world. The International Diabetes Federation (IDF) estimated [1] that DM will be one of the most serious causes of death by 2030, and the number of adults who suffered from DM will rise highly to 642 million by 2040. Diabetic cardiomyopathy (DCM) is one of the main complications of DM, which contributes to the high fatality rate in DCM patients. Myocardial fibrosis is a popular pathological process of DCM.

In previous studies, scientists usually focused on cardiomyocyte in myocardial injury *in vivo* [2, 3]. However, it is a widely recognized myocardial injury, especially myocardial fibrosis, which is closely related to the pathophysiological changes of cardiac fibroblasts (CFs) [4]. Myocardial pathological remodeling involves not only the reactivation of cardiomyocyte death but also CF proliferation and ECM expression. Cardiac fibroblasts are the main components of the heart besides cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells [5]. CFs have the strong proliferation capacity, and the number is about two times of the cardiomyocytes in the heart [6]. Cardiac fibroblasts are the primary cell type responsible for synthesis, deposition, and degradation of extracellular matrix (ECM) proteins. ECM is no longer considered a static support structure for cells, but a dynamic signaling network with the power to influence cells, tissues, and whole organ physiology. Therefore,

ECM proteins play a critical role in the development and maintenance of functional heart tissue, and the changes of cardiac fibroblast function will lead to heart failure. Among the main components of ECM, collagens play the important role in myocardium remodeling. Cardiac fibrosis is a final common pathway in many cardiovascular diseases, which is characterized by the proliferation of CFs and excessive deposition of ECM [7]. Therefore, it is critical to investigate the role and mechanism of CFs on myocardial fibrosis.

Acetaldehyde dehydrogenase 2 (ALDH2) is a member of 19 ALDH gene families. It plays a crucial role in the inhibition of oxidative stress and the detoxification of reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE) [8]. Our previous studies had reported that increasing ALDH2 expression can ameliorate myocardial ischemia/reperfusion injury and diabetes mellitus-induced myocardial injury [9–11]. Other papers also showed that ALDH2 can attenuate cardiac injury induced by toxic metabolites [12], and activation of ALDH2 inhibited AMPK activation, increased the phosphorylation of FOXO3a (Forkhead box O3), and reduced myocardial apoptosis by high glucose-induced myocardial injury [13]. The activation of ALDH2 can resist the excessive production of oxygen radicals caused by various kinds of myocardial injury and apoptosis; however, these studies mainly focused on cardiomyocyte injury and paid few attentions on cardiac fibroblasts. Our previous results showed that myocardial fibrosis happened in a rat DM model with the increases of hydroxyproline, the collagen deposition, and the failure of myocardial systolic and diastolic dysfunction [10]. So, is ALDH2 expressed in myocardial fibroblast? If it is expressed, was ALDH2 downregulated in high glucose-induced myocardial fibroblast injury? And can increasing ALDH2 expression protect myocardial fibroblast against high glucose-induced injury? The underlying mechanisms are still not clearly understood.

So, based on our previous study [9–11], we offered the hypothesis in this study: ALDH2 is expressed in neonate rat cardiac fibroblasts; then, activation of ALDH2 can attenuate high glucose-induced myocardial fibroblast injury. We selected 30 mM glucose to induce neonate rat cardiac fibroblast injury, to observe the expression of ALDH2, and to investigate the likely mechanisms of ALDH2 on cardiac fibroblast injury.

2. Materials and Methods

2.1. Isolation, Primary Culture, and Identification of Cardiac Fibroblasts. The apexes of the hearts were isolated from 1 ~ 3-day-old Sprague-Dawley rats which were obtained from the Experimental Animal Center of Bengbu Medical College (Bengbu, China). All the animal procedures were in accordance with the United States National Institutes of Health Guide and were approved by the Animal Use and Care Committee of Bengbu Medical College.

After washing in precooling D-Hank's solution, the heart tissues were sheared and fully digested (37°C, 5% CO_2, incubated for 7 ~ 8 min). The digestive enzymes consisted of trypsin (0.07%, Beyotime Biotechnology, Shanghai, China), type II collagenase (0.08%, Sigma-Aldrich Co., St. Louis, MO, USA), and DNase I (10 μg/mL, Beijing Solarbio Science & Technology Co. Ltd., Beijing, China). When the tissue started to loosen, precooling DMEM medium (10% FBS) was added to stop the digestion. Cells isolated from the tissue were collected and cultured in DMEM medium (glucose 5.5 mM) containing 10% FBS in an incubator (37°C, 5% CO_2) for 90 min. Vimentin (1 : 200, Boster Biological Technology, Wuhan, China)-positive cells were considered as CFs. As the cells grew to 80% confluence, they were passaged at a ratio of 1 : 2, and the 2nd to 4th passages of cells were used for the following experiments.

2.2. Double-Label Immunofluorescence Staining. ALDH2 expression of CFs was detected by double-label immunofluorescence staining technique. Anti-vimentin antibody (1 : 200, Boster Biological Technology, Wuhan, China) and rabbit anti-ALDH2 antibody (1 : 100, Abcam Co., Cambridge, UK) were used in the experiment operation. Cell slides were incubated with mixed primary antibody overnight at 4°C after blocking with 5% bovine serum albumin (BSA) for 30 min at 37°C. The mixed secondary antibodies were added and incubated in 37°C for the optimized time and dilution. The nuclei of CFs were stained with DAPI for 10 min in 37°C. Fluorescent images were obtained with fluorescence microscope camera (OLYMPUS U-HGLGPS, Japan).

2.3. Identification of ALDH2 Expression in CFs. For identifying whether ALDH2 is expressed in CFs, ALDH2 mRNA and protein expressions were detected by reverse transcription polymerase chain reaction (RT-PCR) and Western blot. Total RNA from CFs was isolated using TRIzol reagent (Invitrogen, Grand Island, NY, USA), and 50 ng of total cDNA was used for PCR analysis with PCR Master Mix (2×) (K0171, Thermo Fisher Scientific Inc., New York, USA), after reverse transcription using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific Inc., New York, USA). The thermal cycling conditions of RT-PCR are as follows: 95°C for 3 min, then 40 cycles of 95°C for 30 sec, 62.5°C for 30 sec, and 72°C for 35 sec, followed by a final extension step at 72°C for 10 min.

2.4. Experimental Grouping. CFs were divided into 7 groups after incubated (37°C, 5% CO_2) in a serum-free DMEM medium for 48 h:

Group 1: Normal group (NG), CFs were cultured with DMEM medium with normal glucose (glucose concentration at 5.5 mM) and treated with the same volume of solvent instead of drug.

Group 2: Normal glucose group + Alda-1 (NG + Alda-1), Alda-1 at 20 μM (the specific agonist of ALDH2, Sigma-Aldrich Co., St. Louis, MO, USA) [14] was added into DMEM medium with normal glucose and cultured for 48 h.

Group 3: High glucose groups (HG), CFs were cultured in DMEM medium with high glucose (glucose

Table 1: Quantitative polymerase chain reaction primers for ALDH2, collagen I, collagen III, and GAPDH.

Gene (accession number)	Sequence	Annealing temperature (°C)	Product (bp)
ALDH2 (NM_032416.1)	Forward 5′-GTG TTC GGA GAC GTC AAA GA-3′ Reverse 5′-GCA GAG CTT GGG ACA GGT AA-3′	62.5	187
Collagen I (NM_053304.1)	Forward 5′-CCA GCG GTG GTT ATG ACT TCA-3′ Reverse 5′-TGC TGG CTC AGG CTC TTG A-3′	59	148
Collagen III (NM_032085.1)	Forward 5′-GGTCACTTTCACTGGTTGACGA-3′ Reverse 5′-TTGAATATCAAACACGCAAGGC-3′	59	201
GAPDH (NM_017008.4)	Forward 5′-ACA GCA ACA GGG TGG AC-3′ Reverse 5′-TTT GAG GGT GCA GCG AAC TT-3′	62	255

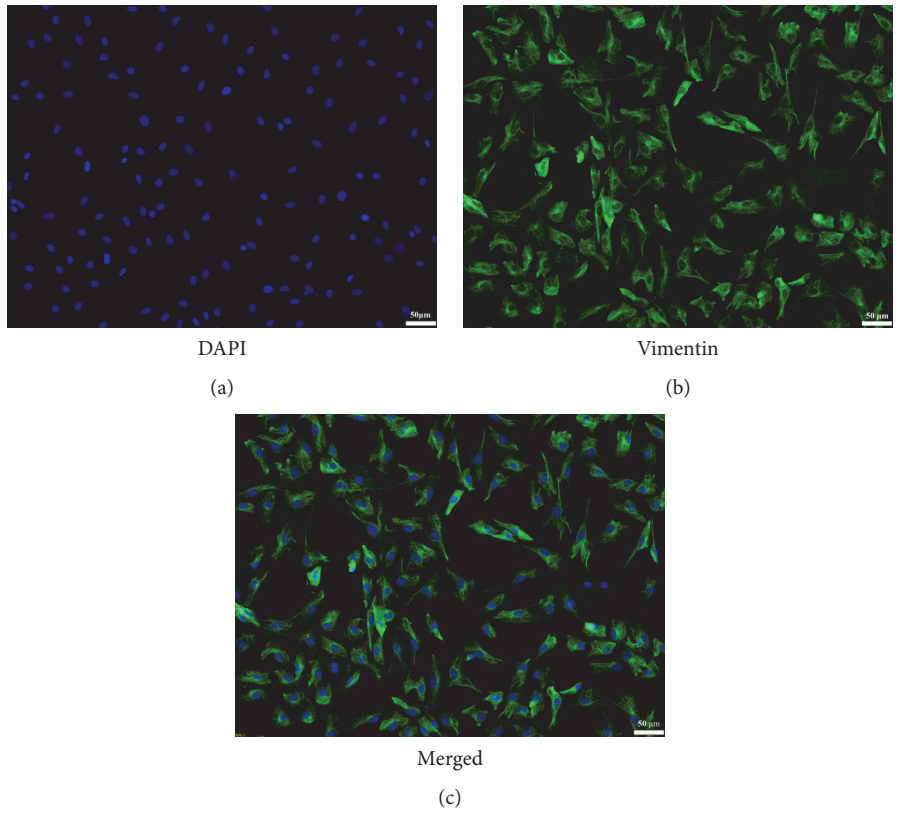

Figure 1: The identification of cardiac fibroblasts by immunofluorescence technique. (a) Nuclei of CFs were stained with DAPI (magnification ×100). (b) The cytoplasm was stained green which showed that CFs can be identified with the positive expression of vimentin (magnification ×100). (c) The merged picture of (a) and (b) (magnification ×100).

concentration at 30 mM) to induce injury for 48 h.

Group 4: HG + Alda-1 group (HG + Alda-1), for observing whether activation of ALDH2 can attenuate HG-induced CF injury, 20 μM Alda-1 was added into DMEM medium with 30 mM glucose and cultured for 48 h.

Group 5: HG + Alda-1 + daidzin (HG + Alda-1 + daidzin), 20 μM Alda-1 and 60 μM daidzin (the specific antagonist of ALDH2, Sigma-Aldrich Co., St. Louis, MO, USA) [15] were added into DMEM medium with 30 mM glucose and cultured for 48 h.

Group 6: HG + daidzin group (HG + daidzin), 60 μM daidzin was added into DMEM medium with 30 mM glucose and cultured for 48 h.

Group 7: Hypertonic group (HPG), for excluding the role of hypertonic, CFs were cultured with DMEM medium with 5.5 mM glucose and treated with 24.5 mM mannitol 48 h.

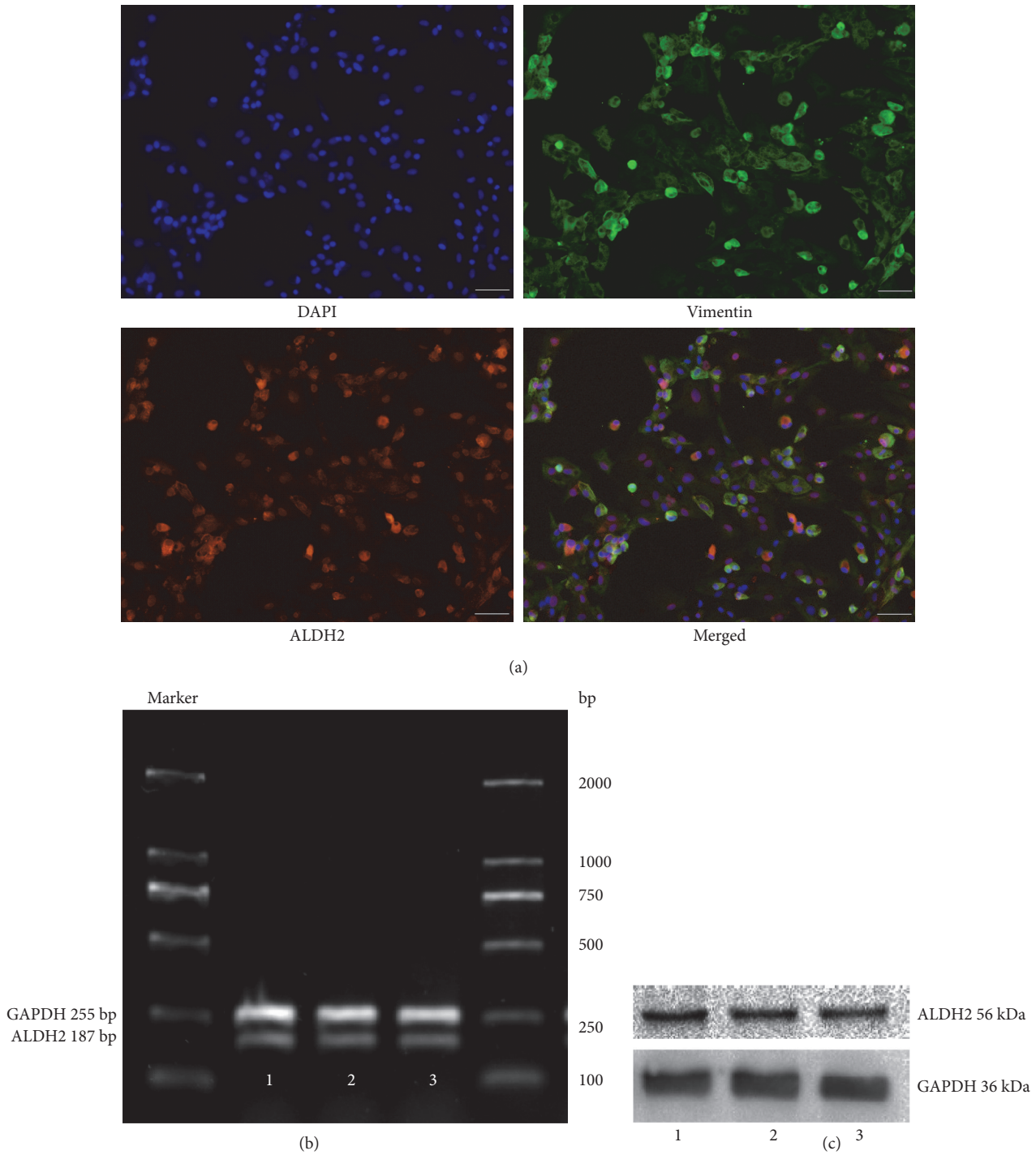

Figure 2: The ALDH2 expression of cardiac fibroblasts detected by double-label immunofluorescence-staining technique (magnification ×100) (a). Red fluorescence-positive cells indicated that ALDH2 was expressed in CFs. The mRNA (b) and protein (c) expressions of ALDH2 were detected by RT-PCR and Western blot in three different batches of CFs. GAPDH was used as a loading control.

2.5. MTT Measurement. MTT measurement was done in all groups. CFs were seeded in 96-well plates at a density of 1×10^6 cells/plate. Cell viability was assessed in seven different groups using MTT assay (Biosharp, Hefei, China) according to the manufacturer. The optical density (OD) values of the cells in each well of different groups were measured at 490 nm.

DHE staining, qRT-PCR, Western blot, and apoptosis measurements were done in six groups excluding hypertonic group.

2.6. ROS Level Detected by DHE. Superoxide production in the CFs was detected by dihydroethidium (DHE, Sigma-Aldrich Co., St. Louis, MO, USA) staining. CFs were seeded

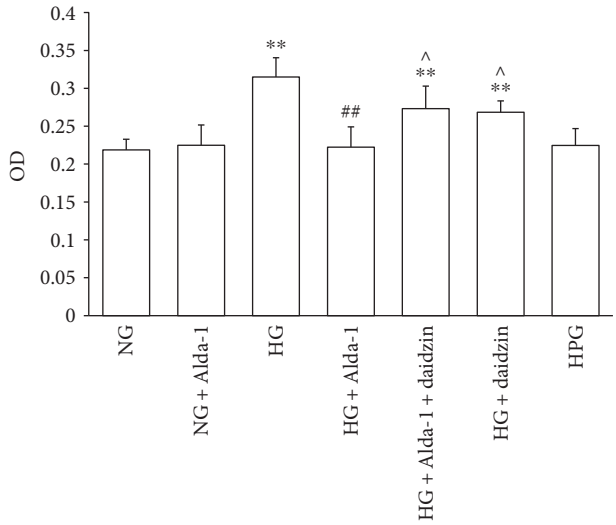

Figure 3: The OD value of CFs in different experimental groups were measured by MTT method. Data were presented as the mean ± SEM ($N = 6$). $^{**}P < 0.01$ versus NG, $^{\#\#}P < 0.01$ versus HG, $^{\wedge}P < 0.05$ versus HG + Alda-1.

in 6-well plates at a density of 1×10^6 cells/plate. The CFs were incubated with 1 μM DHE solution at 37°C for 30 min away from light. Fluorescent images were obtained with fluorescence microscope camera (OLYMPUS U-HGLGPS, Japan) and the mean fluorescence intensity was analyzed with ImageJ software.

2.7. ALDH2 Activity Detection. The ALDH2 activity was assessed using the mitochondrial aldehyde dehydrogenase (ALDH2) activity assay kit (ab115348, Abcam Co., Cambridge, UK). Briefly, the activity is determined by following the production of NADH in the following ALDH2 catalyzed reaction: acetaldehyde + $NAD^+ \rightarrow$ acid + NADH, we determined the activity of ALDH2 by measuring absorbance of acid at 450 nm. All reagents were provided and we conducted the experiment according to the manufacturer's protocol.

2.8. Reverse Transcription Real-Time PCR (qRT-RCR). Total RNA from CFs was isolated using TRIzol reagent (Invitrogen, Grand island, NY, USA), and 50 ng of total cDNA was used for real-time PCR analysis with SYBR® Premix Dimer-Eraser™ Kit (Takara Biotechnology (Dalian) Co. Ltd., Dalian, China) after reverse transcription using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific Inc., New York, USA). The thermal cycling conditions of real-time PCR are as follows: 95°C for 3 sec, then 40 cycles of 95°C for 5 sec, 59°C for 30 sec, and 72°C for 34 sec. The primers we purchased from Sangon Biotech (Shanghai, China) listed in Table 1. Gene expression was normalized to the endogenous control (GAPDH mRNA), and the amount of target gene mRNA expression in each sample was expressed relative to that of the control. The details of Western blot were explained in the following text.

2.9. Western Blot Analysis of ALDH2 and 4-HNE. CFs in each group were collected and homogenized in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) (add PMSF 0.1 mM) for 1 hour on ice. The lysates were centrifuged at 10,000 rpm and 4°C for 15 min, and the supernatants were used for Western blot after protein quantification. Proteins were separated by SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes [16]. After blocking by nonfat milk for 2.5 h, immunoblotting was performed with the following antibodies: anti-GAPDH antibody (36 kDa, 1 : 1000, Boster Biological Technology, Wuhan, China) as a control for loading, rabbit anti-ALDH2 antibody (56 kDa, 1 : 3000, Abcam Co., Cambridge, UK), and 4-HNE (70 kDa, 1 : 2000, Abcam Co., Cambridge, UK). Detection and quantification were performed by ECL with horseradish peroxidase- (HRP-) linked anti-rabbit IgG (1 : 10,000, Boster Biological Technology, Wuhan, China). Densitometric quantification of antibody-specific dots was performed with ChemiDoc™ XRS+ System and analyzed with Tanon software (version 4.2.1).

2.10. Flow Cytometry. Apoptosis rate was detected by Annexin V and propidium iodide double staining method through flow cytometry by Annexin V-FITC Apoptosis Detection Kit (Kaiji Biological Engineering Co. Ltd., Nanjing, China). CFs in each group were collected and resuspended in 500 μL binding buffer. CFs were labeled with Annexin V-FITC and propidium iodide (PI) and then incubated for 15 min in the dark. All samples were analyzed by flow cytometry.

2.11. Statistical Analyses. All data analyses were performed using SPSS software (version 16.0) and expressed as means ± SEM. One-way ANOVA analysis (Newman-Keuls for comparisons of multiple means) was used for statistical analyses. P values <0.05 were considered as statistically significant.

3. Results

3.1. The Identification of CFs. Vimentin is a kind of intermediate fiber in interstitial cells, which is an integral part of the cytoskeleton. It maintains the integrity of the cells. It is reported that cardiac fibroblasts can be identified with the positive expression of vimentin. The results showed that vimentin-positive cells with green fluorescence were considered highly purified cardiac fibroblasts (Figure 1).

3.2. The Detection of ALDH2 in CFs. For identifying whether ALDH2 is expressed in CFs, double-label immunofluorescence staining technique was used to observe cellular localization of ALDH2. RT-PCR and Western blot methods were used, respectively, to measure ALDH2 expression at mRNA and protein level in three different batches of CFs. ALDH2-positive cells with red fluorescence indicated that ALDH2 was expressed in CFs (Figure 2(a)). Meanwhile, ALDH2 at mRNA (Figure 2(b)) and protein (Figure 2(c)) levels were expressed in cardiac fibroblasts.

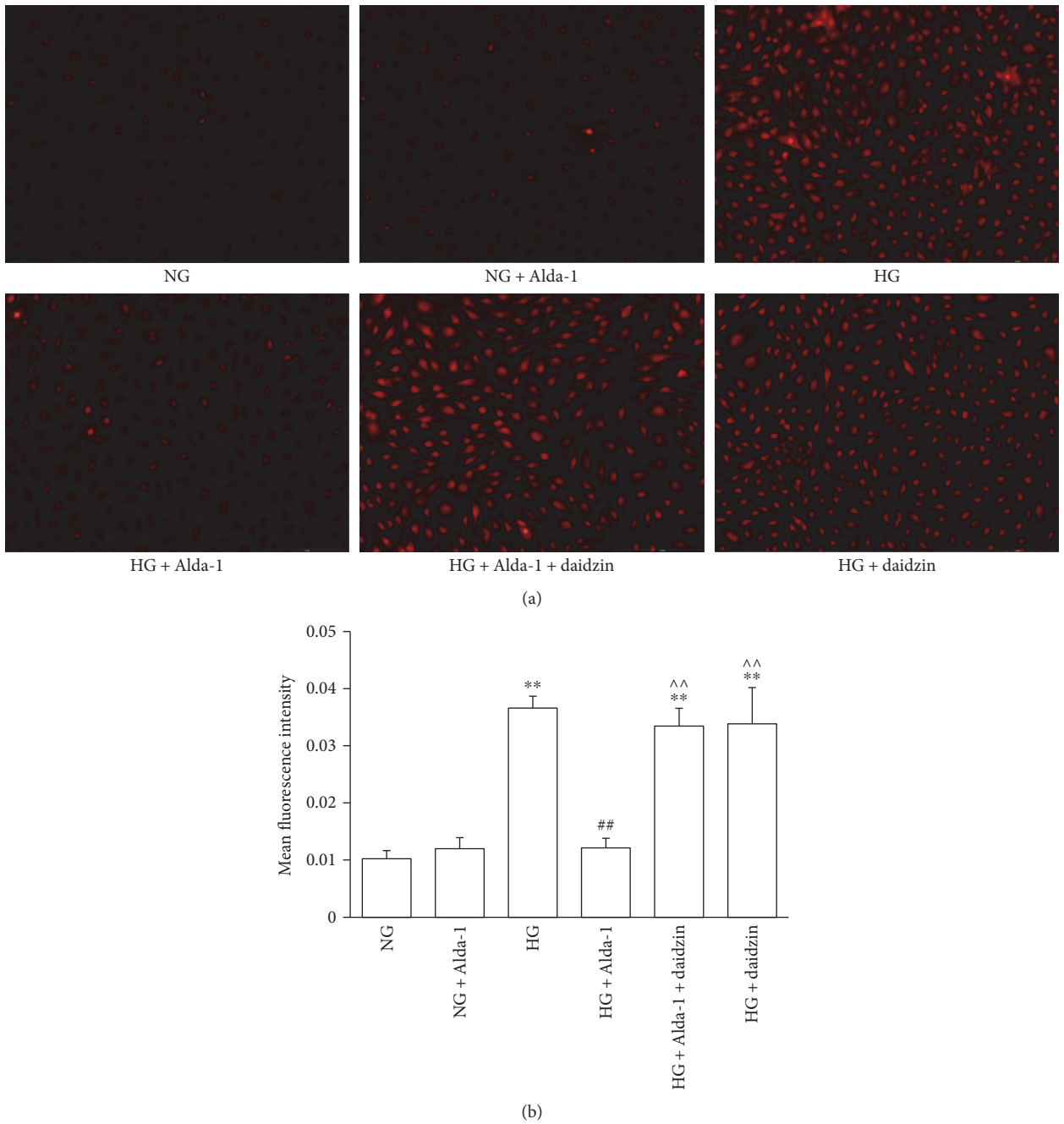

Figure 4: ROS level of CFs in different experimental groups. (a) Intracellular ROS accumulation in different groups stained by DHE. Representative pictures were shown. (b) Mean fluorescence intensity was detected by ImageJ software. Data were presented as the mean ± SEM ($N = 3$). $^{**}P < 0.01$ versus NG, $^{\#\#}P < 0.01$ versus HG, $^{\wedge\wedge}P < 0.01$ versus HG + Alda-1.

3.3. MTT Measurement. There was no significant difference in cell viability and proliferation of CFs among normal group (NG), NG + Alda-1, and HPG groups, so HPG intervention was not used in the mechanism research. Cell viability and proliferation of CFs in HG, HG + Alda-1 + daidzin, and HG + daidzin were higher than those of cells in NG ($P < 0.01$). When compared with HG, the viability and proliferation in HG + Alda-1 were inhibited ($P < 0.01$). The viability and proliferation were increased ($P < 0.05$) in HG + Alda-1 + daidzin and HG + daidzin groups compared with HG + Alda-1 (Figure 3).

3.4. The Levels of ROS by DHE Staining. Compared with NG group, there was no change of DHE fluorescence intensity in NG + Alda-1 group, but DHE fluorescence intensity was enhanced significantly in HG group. When compared with HG group, DHE fluorescence intensity was obviously weak in HG + Alda-1 group and was stronger in HG + Alda-1 + daidzin and HG + daidzin groups (Figures 4(a) and 4(b)).

3.5. Changes of ALDH2 Activity in Each Group. ALDH2 activity was assessed in primary CFs isolated from neonatal rats (Figure 5). The results showed there was no significant

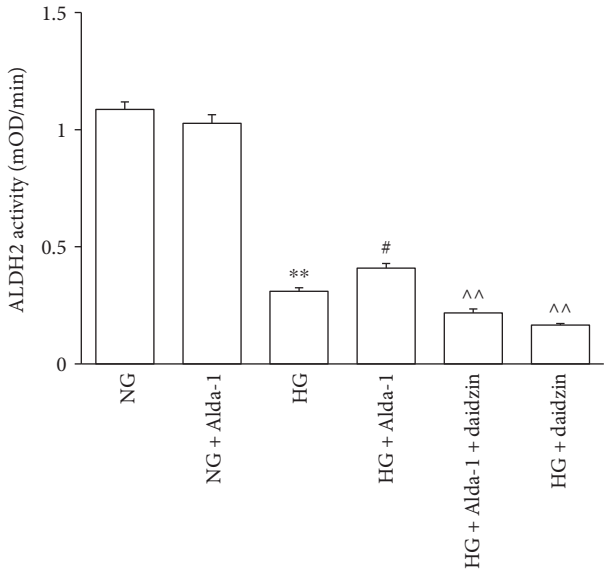

Figure 5: ALDH2 activity in different experimental groups. Data were presented as mean ± SEM ($N = 6$). $^{**}P < 0.01$ versus NG, $^{\#}P < 0.05$ versus HG, $^{\wedge\wedge}P < 0.01$ versus HG + Alda-1.

difference of ALDH2 activity between NG and NG + Alda-1 groups. Compared with NG group, the activity of ALDH2 was decreased in HG group. When compared with HG group, the activity of ALDH2 was increased in HG + Alda-1 group. The activity of ALDH2 was reduced significantly in HG + Alda-1 + daidzin and HG + daidzin groups compared with HG + Alda-1 group.

3.6. Changes of the Expressions of Collagen I and Collagen III at mRNA Level. There was no significant difference of the expressions of collagen I and collagen III at mRNA level in NG and NG + Alda-1 groups. When compared with NG group, the mRNA expressions of collagen I and collagen III were increased ($P < 0.01$) in HG group. However, collagen I and collagen III mRNA expressions in HG + Alda-1 group were decreased compared with HG group ($P < 0.01$). When compared with HG + Alda-1 group, the mRNA expressions of collagen I and collagen III in HG + Alda-1 + daidzin and HG + daidzin groups were increased ($P < 0.05$) (Figures 6(a) and 6(b)).

3.7. Changes of the Expressions of 4-HNE and ALDH2 at Protein Level. There was no significant difference of 4-HNE and ALDH2 in NG and NG + Alda-1 groups. When compared with NG group, ALDH2 protein expression in HG group was decreased ($P < 0.01$), while 4-HNE expression was increased ($P < 0.01$). The protein expression of ALDH2 in HG + Alda-1 group was increased ($P < 0.01$), while the protein expression of 4-HNE was decreased ($P < 0.01$) compared with HG group. When compared with HG + Alda-1 group, the protein expression of ALDH2 were decreased ($P < 0.01$), and the protein expression of 4-HNE was increased ($P < 0.05$) in HG + Alda-1 + daidzin, and HG + daidzin groups were decreased ($P < 0.01$) while the expression of 4-HNE was increased ($P < 0.05$) (Figures 7(a), 7(b), 7(c), and 7(d)).

3.8. Apoptosis of Cardiac Fibroblasts. The CF apoptosis was determined by Annexin V and PI double staining method using flow cytometry. The results indicated that there was no variation of apoptosis rate in NG and NG + Alda-1 group. The apoptosis rate in HG group was increased compared with NG group ($P < 0.01$). The apoptosis rate of in HG + Alda-1 group was decreased compared with HG group ($P < 0.01$). Compared with HG + Alda-1 group, the apoptosis rates in HG + Alda-1 + daidzin and HG + daidzin groups were increased significantly ($P < 0.01$) (Figures 8(a) and 8(b)).

4. Discussion

In this study, we characterized the expression and function of ALDH2 on high glucose-induced CF changes. Firstly, double-label immunofluorescence staining, RT-PCR, and Western blot results provided the first evidence that the location of ALDH2 in CFs, which indicated ALDH2, could be necessary in CF function. We observed ALDH2 protein was downregulated accompanied with fibrosis and apoptosis when CFs were cultured with high glucose. Activation of ALDH2 with the specific agonist Alda-1 could restrain the proliferation of CFs cultured with high glucose, reduce the release of ROS and 4-HNE protein expression, decrease oxidative stress overload as well as the expressions of collagen I and collagen III, reverse myocardial fibrosis, and attenuate CFs apoptosis. ALDH2 activity and protein expression were increased at the same time. Taken together, our data indicated that ALDH2 played a protective role in high glucose-induced cardiac fibroblast injury model.

ALDH2 is widely expressed in the heart, brain, liver, kidney, and lung and involved in the occurrence and development of illnesses. Previous studies had shown that ALDH2 attenuated diabetes-induced myocardial injury [17], but is ALDH2 expressed in cardiac fibroblasts? Can ALDH2 protect myocardium against fibrosis? It is rarely reported. We have verified that when treated with a low concentration of ethanol, the nonselective agonist of ALDH2, myocardial fibrosis was improved in diabetic rat [18]. As we know, overproduction of cardiac fibroblasts is one of the important reasons of myocardial fibrosis. In the light of our own and others' research, we speculate that ALDH2 may be expressed in cardiac fibroblasts and is involved in the occurrence of myocardial fibrosis. As we expected, our results showed that ALDH2 was expressed in CFs. It is beneficial for us to investigate the mechanisms of diabetes-induced myocardial fibrosis intensively.

ALDH2 has the protective effects on the various types of cardiovascular injury, such as microvascular injury induced by diabetes, myocardial injury in diabetic rats, and cardiomyocyte injury induced by high glucose [19–22]. Pretreatment with the specific agonist of ALDH2 Alda-1 can improve myocardial ischemia/reperfusion injury in rats by upregulating the expression of ALDH2 [23] and play an important protective role in high glucose-induced

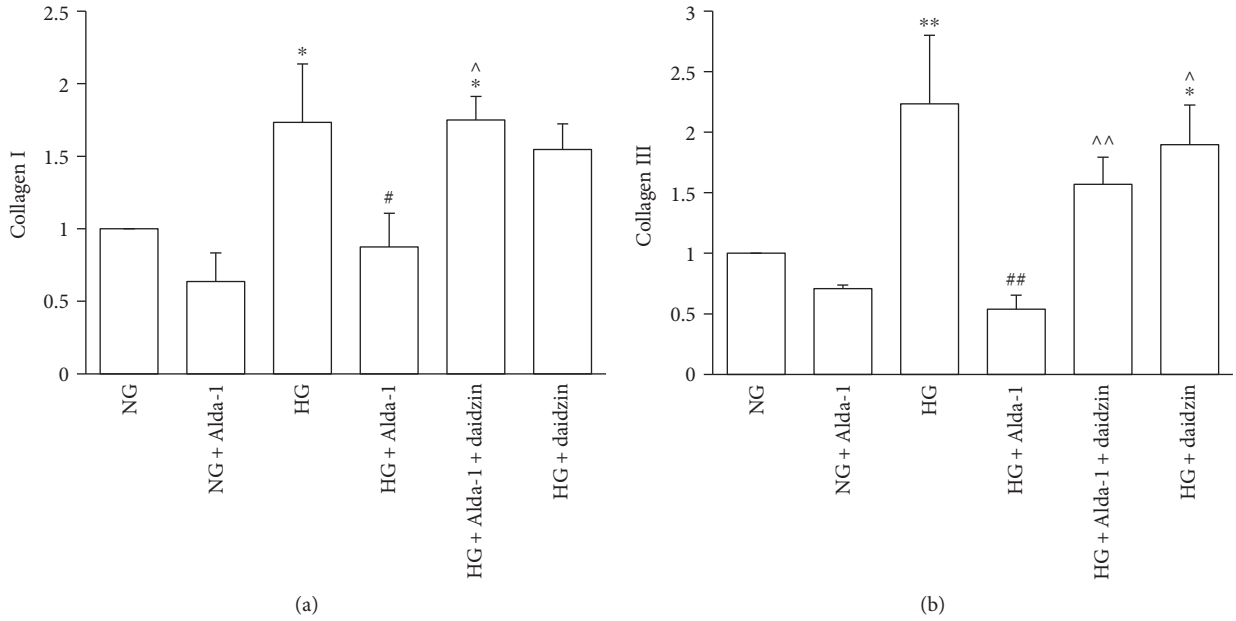

Figure 6: Expressions of collagen I and collagen III at mRNA level in the different experimental groups. mRNA levels of collagen I (a) and collagen III (b) were quantified by real-time-PCR analysis in the CFs of each experimental group. GAPDH was used as a loading control. Data were presented as the mean ± SEM ($N = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus NG, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HG, $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ versus HG + Alda-1.

myocardial cell injury [13]. Activation of ALDH2 can reduce myocardial fibrosis in diabetic rats and inhibit the expression of JNK (c-Jun N-terminal kinase) which is important in cell proliferation, differentiation, apoptosis, fibrosis, and so on [18]. In this study, we have identified that ALDH2 was expressed in CFs, so we further observe whether activation of ALDH2 also plays a protective effect in high glucose-induced CF injury.

Under normal physiological circumstances, the production and scavenging ability of ROS in intracellular environment are balanced dynamically. Oxidative stress occurred when the production of ROS is far greater than elimination, causing oxidative damage to the DNA and abnormal expression of proteins, finally contributing to cell injury. In recent years, studies showed that the occurrence of myocardial fibrosis in diabetic rats is closely related to oxidative stress. The overproduction of ROS induced by high glucose can attack the polyunsaturated fatty acid (PUFA) of the phospholipid bilayer on the cell membranes, lead to lipid peroxidation, and result in enhanced 4-HNE, an aldehyde product of membrane lipid peroxidation [24]. 4-HNE is one of the representative reactive aldehydes which had been detected in several diseases such as atherosclerosis, diabetes, Parkinson's disease, and cancer [25, 26]. High concentration of 4-HNE can induce cell apoptosis, influence the cell signal transduction, and have a cytotoxic effect [27–29]. ROS has been considered to be a key factor in the development of diabetes and other diseases; the formation of 4-HNE and 4-HNE-protein conjugation had become a marker of oxidative stress in tissues or cells [24, 30–32]. Inhibiting ROS and 4-HNE production can reduce myocardial fibrosis and improve cardiac function in rats with diabetes mellitus [28]. ALDH2 is a key enzyme that metabolizes acetaldehyde and other aldehyde metabolites such as 4-HNE to nontoxic products [33]. In our study, ROS level and 4-HNE protein expression were increased in high-glucose cultured CFs, suggesting high glucose-induced overproduction of ROS and 4-HNE. When given with the specific agonist of ALDH2 Alda-1 in HG-treated CFs, ROS level and 4-HNE protein expression were decreased, while ROS level and 4-HNE protein expression were increased in CFs after treated with the specific ALDH2 antagonist daidzin. These results suggested that ALDH2 played a key role in high glucose-induced oxidative stress, and activation of ALDH2 can eliminate the overproduction of ROS and 4-HNE, then protect CFs against high glucose-induced cell injury.

Fibrosis is an important pathological change in high glucose-induced cardiac fibroblast injury. The main reason of myocardial fibrosis is the accumulation of collagen which disorders the structure of the heart. Collagen I and collagen III are the main collagen components, and among them, collagen I accounts for about 80%, it determines myocardial stiffness, and collagen III accounts for about 12%, which determines myocardial compliance; the balance of collagen I and III plays a significant role in maintaining normal physiological function [34–36]. The oversynthesis of collagen I and collagen III, especially collagen I, may be the main pathological change in myocardial fibrosis. The increased collagen I and collagen III were involved in the myocardial collagen network remodeling, as well as in myocardial fibrosis caused by diabetes mellitus [36]. There was no difference in the expression of ALDH2, collagen I, and collagen III when CFs were treated with Alda-1 compared with control group, it suggested that Alda-1 had no obvious role in ALDH2 expression when CFs were in normal situation. However, when CFs were treated with high glucose,

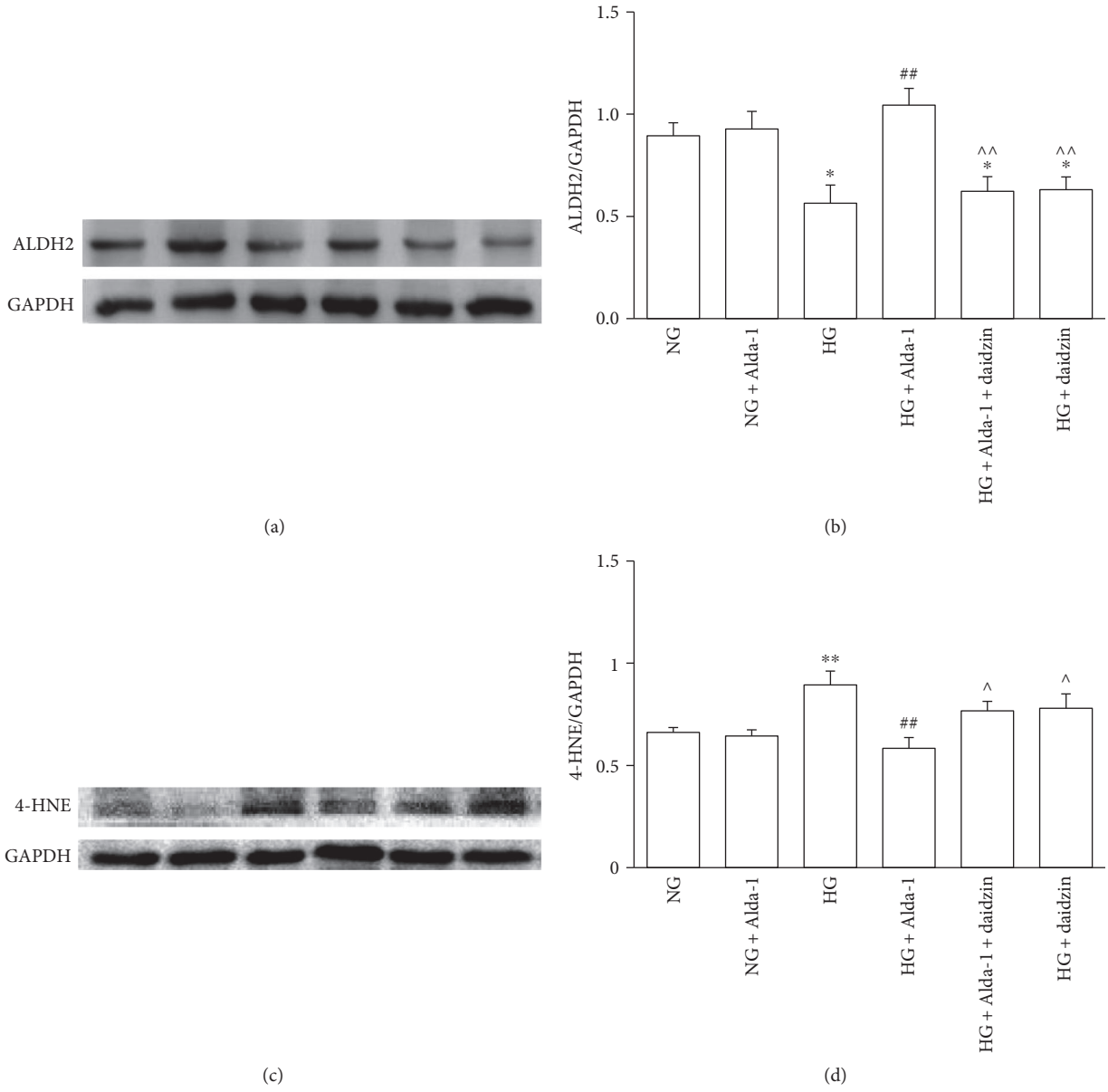

Figure 7: The changes of ALDH2 and 4-HNE protein levels in different experimental groups. (a) Representative blots of ALDH2 and GAPDH in CFs. (b) ALDH2 protein levels in CFs normalized by GAPDH levels and all the data were presented as mean ± SEM ($N = 8$). (c) Representative blots of 4-HNE and GAPDH in CFs. (d) 4-HNE protein levels in CFs normalized by GAPDH levels and all the data were presented as mean ± SEM ($N = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus NG, $^{\#\#}P < 0.01$ versus HG, $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ versus HG + Alda-1.

accompanied by the decreases of ALDH2 activity and protein expression, collagen I and collagen III mRNA expressions were increased. It suggested that high glucose can induce the happening of CF fibrosis. When CFs were treated with high glucose and ALDH2 agonist Alda-1, ALDH2 activity and expression were increased while the collagen I and collagen III mRNA expressions were decreased, it suggested that Alda-1 promote the expression of ALDH2, and the increase of ALDH2 can reverse myocardial fibrosis. After treated with daidzin, the ALDH2-specific antagonist, ALDH2 activity and expression were decreased; the synthesis of collagen I and collagen III were increased, aggravating myocardial fibrosis, and further verified the key role of ALDH2. Decreasing ALDH2 expression can induce myocardial fibrosis.

During cardiac fibrosis, complex molecular mechanisms, which play the critical roles in regulating cardiac fibroblast apoptosis, have been shown to be closely related to the occurrence of fibrosis. Apoptotic cells act as the drivers of fibrotic process, it may act directly on CFs, enhancing cellular proliferation and profibrotic phenotypes. High levels of apoptosis are either initiators or perpetuators of the fibrotic response seen in lung fibrosis, liver fibrosis, and chronic myocardium fibrosis accompanied by deposition of extracellular matrix, synthesis of collagen, and fibroblast proliferation [37–39]. High glucose also induced the happening of apoptosis in CFs, in which oxidative stress is the key inducement. The major mechanisms of oxidative stress-induced apoptosis may be as follows [40]: (1) the increased level of ROS leads

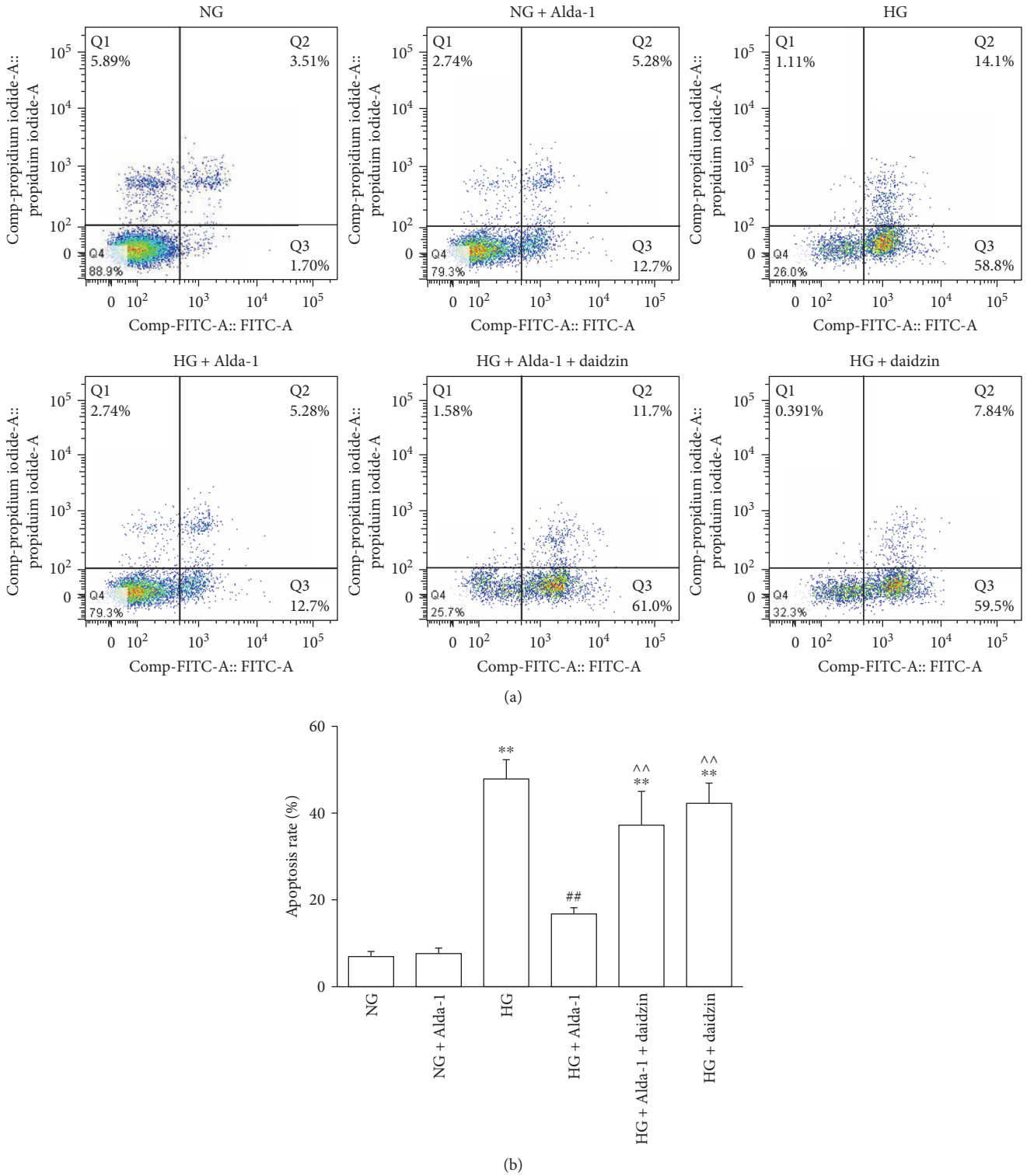

Figure 8: Apoptosis of CFs in different experimental groups. (a) CFs were labeled with Annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Representative pictures were shown. Q1, Q2, Q3, and Q4 represent necrosis, early-stage apoptosis, late-stage apoptosis, and normal cells, respectively. (b) Q2 and Q3 were chosen to analyze the change of apoptosis rate in different experimental groups. Data were presented as mean ± SEM ($N = 3$). $^{**}P < 0.01$ versus NG, $^{\#\#}P < 0.01$ versus HG, $^{\wedge\wedge}P < 0.01$ versus HG + Alda-1.

to NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells, which was involved in the control of a large number of cellular processes, such as immune and inflammatory responses, cellular growth and development, and apoptosis) activation, which combines with apoptosis-related genes such as c-myc gene to promote transcription

and apoptosis; (2) DNA is damaged by ROS, which activates the P53 gene, leading to apoptosis; (3) the increased ROS can directly or indirectly damage the mitochondrial membrane, which leads to the increase of the permeability of the membrane and the activation of the apoptotic protease; (4) ROS activates the MAPK (mitogen-activated protein kinases) signal transduction pathway and then regulates cell proliferation, gene expression, differentiation, mitosis, and cell survival, which eventually activates caspase cascade and induces apoptosis. We have observed that ALDH2 can decrease high glucose-induced overproduction of ROS in a CF model. If ROS is close with apoptosis, can ALDH2 attenuate the occurrence of apoptosis? Our results showed that the apoptosis rate of CFs was increased obviously when cultured with high glucose, while the apoptosis rate was decreased when added Alda-1 in HG group, it suggested that ALDH2 can inhibit the occurrence of apoptosis. When ALDH2 activity was inhibited, the apoptosis rate was increased in HG + Alda-1 + daidzin group, and more verifiably, ALDH2 can regulate the happening of apoptosis. Combined with these data, we speculated that ALDH2 attenuated the damage degree of CF fibrosis through inhibiting apoptosis happening.

In summary, we reported for the first time that ALDH2 is expressed in cardiac fibroblasts. Then, we reported that high glucose can increase oxygen stress reaction, decrease ALDH2 activity and expression, and induce cardiac fibroblast apoptosis and fibrosis. Especially, we reported that the activation of ALDH2 may ameliorate high glucose-induced cardiac fibroblast fibrosis through decreasing oxidative stress and apoptosis. The study may be beneficial to remedy myocardial fibrosis induced by diabetes and other diseases. The intracellular signal transduction mechanisms remain to be further explored.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Platelet Carbonic Anhydrase II, a Forgotten Enzyme, May Be Responsible for Aspirin Resistance

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Background. Thromboembolic events constitute a major health problem, despite the steadily expanding arsenal of antiplatelet drugs. Hence, there is still a need to optimize the antiplatelet therapy. *Objectives*. The aim of our study was to verify a hypothesis that there are no differences in platelet proteome between two groups of healthy people representing different acetylsalicylic acid (aspirin) responses as assessed by the liquid chromatography/mass spectrometry (LC/MS) technique. *Patients/Methods*. A total of 61 healthy volunteers were recruited for the study. Physical examination and blood collection were followed by platelet-rich plasma aggregation assays and platelet separation for proteomic LC/MS analysis. Arachidonic acid- (AA-) induced aggregation (in the presence of aspirin) allowed to divide study participants into two groups aspirin-resistant (AR) and aspirin-sensitive (AS) ones. Subsequently, platelet proteome was compared in groups using the LC/MS analysis. *Results*. The LC/MS analysis of platelet proteome between groups revealed that out of all identified proteins, the only discriminatory protein, affecting aspirin responsiveness, is platelet carbonic anhydrase II (CA II). *Conclusions*. CA II is a platelet function modulator and should be taken into consideration as a cardiovascular event risk factor or therapeutic target.

1. Introduction

Despite the steadily expanding arsenal of antiplatelet agents, thromboembolic events are still common in clinical practice [1]. Acetylsalicylic acid (ASA, aspirin), widely used in the high-cardiovascular-risk population, is in many cases ineffective. This phenomenon, called aspirin resistance (AR), remains still an important clinical problem. Its pathogenesis and incidence persist the subject of numerous studies and controversies [2–5]. ASA exerts its anti-aggregatory effect mostly by inhibiting the platelet COX-1 and subsequently thromboxane formation from the arachidonic acid cascade. Aspirin resistance, at molecular level, may be attributed, that is, to changes in efficacy of COX-1 acetylating by ASA [6]. Studies focusing on the AR phenomenon defined in this way do not precise the time frame between the last aspirin dose and blood sampling [6, 7].

On the other hand, ASA treatment failure may result from factors that are COX-1 independent, including noncompliance [7], increased platelet turnover [2], and platelet COX-1 resynthesis [8], and factors increasing platelet reactivity. Up to date, there are several papers describing factors, which expression increases during ASA therapy and which are associated with the AR phenomenon. Studies by Voora et al. show that following 4 weeks of ASA treatment (325 mg/day), the platelet sensitivity to ADP, epinephrine, and collagen significantly increases despite sufficient COX inhibition [9]. The authors introduced a term "aspirin response signature," which is a set of coexpressed genes assessed in peripheral blood during ASA treatment that are

strongly correlated with COX1-independent platelet function and represent increased risk of myocardial infarction and death [10].

Furthermore, Massimi et al. proved that ASA in both *in vitro* cell lines [11, 12] and *in vivo* platelets (over two months of treatment) enhances expression of the multidrug resistance protein 4 (MRP4), high concentration of which is associated with greater TxB2 synthesis, and subsequently more intense collagen-induced aggregation [13]. The platelet MRP4 might be also induced by other nonsteroidal anti-inflammatory drugs [14]. The presence of factor enhancing the "on aspirin" arachidonic acid induced aggregation (exclusively limited to COX-1 pathway) verified by Floyd et al. who have found that truly resistant subjects are characterized by higher platelet expression of glycoprotein IIIa (GPIIIa) following 28 days of ASA treatment (300 mg/d) [15].

Noteworthy, in some studies there is observed interindividual heterogeneity in platelet *in vitro* response to low ASA doses [15]. The question arises if, beside inducible factors limiting ASA chronic treatment, there are constitutively present factors limiting platelet responsiveness to ASA at baseline.

A novel approach—the use of techniques analyzing the functional proteome—should allow to define factors determining individual variability of platelet activity. By analyzing the platelet proteome in patients with ASA resistance, we intended to define novel mechanisms limiting platelet responsiveness to acetylsalicylic acid.

Therefore, the aim of the study was to verify the hypothesis regarding the lack of differences in platelet proteome between groups with different sensitivities to aspirin, as assessed by the liquid chromatography/mass apectrometry (LC/MS) technique.

2. Material and Methods

2.1. Bioethics Statement. All experiments were conducted and approved in accordance with the guidelines of the local Bioethics Committee and adhered to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects (revised: November 13, 2001; effective: December 13, 2001). All participants provided their written consent to participate in the study. The written consent forms had been previously approved by the ethics committee.

2.2. Recruitment and Examination. A total of 61 clinically healthy volunteers (at the age of 18–60 years) were enrolled to the study. Exclusion criteria were diabetes, hypertension, chronic and acute inflammatory diseases, mental disorders, malignancies, allergy to nonsteroidal anti-inflammatory drugs (NSAID), or the use of NSAID one week prior to the examination.

All participants underwent standard physical examination, and their blood was collected nontraumatically from the antecubital vein using a Sarstedt S-Monovette® system (Sarstedt AG & Co., Nümbrecht, Germany). Study participants were divided into two groups according to the aspirin response (the presence of platelet aggregation in response to arachidonic acid (AA) following an *in vitro* incubation with a fixed dose of ASA) (Figure 1).

At first, baseline characteristics of the groups were compared: then, the presence of differences in the blood test results (including cardiovascular risk factors and markers of endothelial and platelet function) was verified, which was subsequently followed by an analysis of differences in platelet proteome between aspirin-resistant (AR) and aspirin-sensitive (AS) subjects.

2.3. Aggregometry. Aggregation was performed in platelet-rich plasma using a 2-channel optical aggregometer (Chrono-log 490-2D, Chrono-Log, Pennsylvania, USA), and platelet response to arachidonic acid with and without aspirin was tested simultaneously. The aggregation discriminating the groups was performed using arachidonic acid (at 1 mM of final concentration) added to platelet-rich plasma (PRP) in 5 minutes following its incubation with acetylsalicylic acid (final concentration 30 μM, for 5 minutes). Since agonist application, the reaction progress was recorded for 6 minutes using dedicated software (Aggro/link, Chrono-Log, Pennsylvania, USA). The aggregation results were calculated according to the manufacturer's instruction by the Chronolog Software and are expressed as the area under the aggregation curve (AUC), which equals zero for no aggregation was calculated during the course of aggregation protocol (from agonist addition until the termination of aggregation at the end of the sixth minute).

A complete inhibition of the aggregation by ASA was the criterion for diagnosing aspirin resistance. The experiment was performed at a constant temperature of 37°C.

Also, control aggregations were performed using other final concentrations of agonist and antagonist: AA 0.5 mM and Lys-ASA 0.03 mM and AA 1 mM and Lys-ASA 0.05 mM. Additionally, in cases when Lys-ASA at 0.05 mM of final concentration was insufficient to prevent the AA-induced aggregation, a test with Lys-ASA at 0.1 mM of final concentration and AA at 1 mM of constant concentration was performed.

2.4. Platelet Preparation for Proteomic Analyzes. The whole blood was supplemented with prostacyclin (PGI_2) at the final concentration of 0.06 μg/ml, and centrifuged for 20 minutes at 230 ×g at 21°C in order to obtain PRP. Subsequently, PRP was supplemented with PGI_2 (final concentration 0.3 μg/ml) and centrifuged for 10 minutes at 1000 ×g at 21°C. The plasma was discarded, and the platelet pellet was gently washed three times with 1 ml of Tyrodes-HEPES buffer pH 7.4 ml. Rinsed platelets were suspended in 4 ml of Tyrodes-HEPES buffer pH 7.4 supplemented with $CaCl_2$ (final concentration 1 mM). Resulted suspension was immediately analyzed for platelet count and contamination with WBC i RBC (Sysmex device, Clinical Laboratories Department, University Hospital, Wrocław, Poland).

The pure PLT suspension was adjusted with Tyrodes-HEPES buffer pH 7.4 containing $CaCl_2$ to a final concentration of 2.5×10^8/ml. Samples containing platelets in amounts of 2.5×10^8 and 7.5×10^8 cells were preserved for further proteomic analysis. The samples were obtained

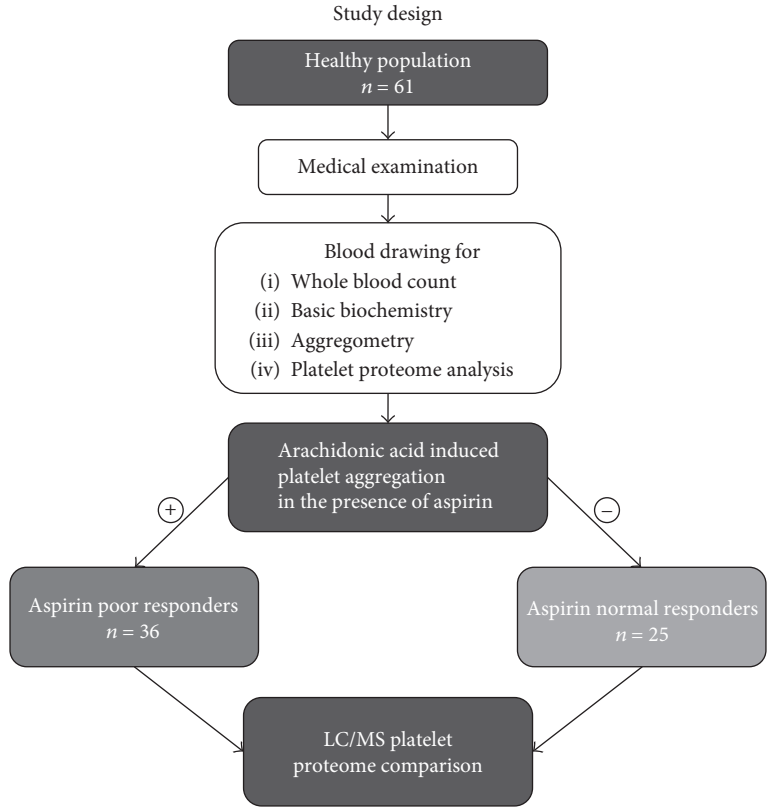

Figure 1: Study protocol.

by centrifugation of the suspension of known concentration for 5 min, 10000 ×g at 4°C, and stored at −80°C until proteomic analyses.

Aggregations of separated platelets in response to collagen were also performed. A 500 μl of platelet suspension (2.5×10^8/ml) was substituted with collagen (final concentration 5 μg/ml), and aggregation was conducted for 6 min and then immediately stopped by placing cuvettes on ice. Pellet and supernatant were separated by consecutive centrifugation, for 5 min at 10000 ×g and 4°C. Obtained material was stored till proteomic analyses at −80°C.

2.5. Liquid Chromatography/Mass Spectrometry (LC/MS). All used reagents were from Sigma, unless otherwise specified. Platelet proteins were extracted by incubation of platelets in 1% sodium deoxycholate, 10 mM TrisHCl pH 8, with the addition of 0.1% sodium dodecyl sulfate, followed by sonication, and clarified by centrifugation (Eppendorf Minispin, 10 min, 12100 g). Protein concentration was measured, and proteins were reduced with 50 mM phosphine, alkylated with 200 mM thiosulfonate, and digested overnight at 37°C with modified trypsin (V5111, Promega). The digestion reaction was quenched by the addition of 2 μl of 10% trifluoroacetic acid. The concentration of the digested peptides was determined by the Direct Detect Method by Millipore. Sample volume corresponding to 5 μg of digested platelet proteins was subjected for proteomic processing, during which peptides were separated by nano-HPLC C-18 column (nanoACQUITY Symmetry® BEH C18, Waters 186003545) using an acetonitrile gradient (5–35% in 180 min) in the presence of 0.1% formic acid with a flow rate of 250 nl/min. The chromatographic column outlet was directly coupled to the ESI-LTQ-Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA, USA) and was operating in the MS (the measurement of the masses of the peptides) and MS/MS (peptides fragmentation) in data-dependent acquisition. The raw data was processed using Mascot Distiller followed by analysis with Mascot software (Matrix Science), using database Swiss-Prot, with taxonomy restricted to *Homo sapiens* [16]. Peptides with a Mascot score exceeding the threshold value corresponding to <1% FDR were considered to be positively identified. Label-free quantization was performed as described previously [17]. The lists of identified proteins were analyzed using Diffprot software [18].

2.6. Endothelial and Platelet Activation Markers. Plasma concentrations of sP-selectin/CD62P and PAI-1 were determined by a sandwich enzyme immunoassay technique, using commercial ELISA kits (Cat: BBE6 and DSE100, R&D Systems Europe Ltd., UK) with a sensitivity of 0.5 ng/ml, according to the manufacturer's instructions. The optical density 450/620 nm was measured with a BioTek Absorbance Microplate Reader with software Gen5. The coefficient of variation (CV) intra-assay %CV was calculated as the ratio of the pooled standard deviation from all samples (each was analyzed in triplicate) and the overall mean and then multiplied by 100. Interassay %CV refers to assay-to-assay consistency that was calculated using the pooled standard deviation

Table 1: Baseline demographic characteristics and biochemical stratification of cardiovascular risk in subgroups separated according to the ASA response.

—	Aspirin resistant (AR) (mean ± SEM)	Aspirin sensitive (AS) (mean ± SEM)	p
N	*36–*	*25–*	
Women [%]	22 (61%)	5 (20%)	
Age [y]	30.28 ± 1.74	29.16 ± 1.81	0.41
BMI [kg/m^2]	22.87 ± 0.61	24.11 ± 0.67	0.10
WBC [k/μl]	5.77 ± 0.22	5.66 ± 0.26	0.80
RBC [mln/μl]	4.68 ± 0.08	4.94 ± 0.09	0.02
Hemoglobin [g/dl]	13.96 ± 0.25	14.74 ± 0.30	0.03
Hematocrit [%]	40.12 ± 0.64	42.12 ± 0.69	0.05
MCV [fl]	85.89 ± 0.51	86.10 ± 0.67	0.82
MCH [pg]	29.86 ± 0.20	30.09 ± 0.35	0.46
MCHC [g/dl]	34.77 ± 0.15	34.95 ± 0.22	0.54
PLT [k/μl]	236.72 ± 9.98	213.76 ± 9.32	0.12
PDW [fl]	13.79 ± 0.37	13.55 ± 0.46	0.50
Glucose [mg/dl]	83.39 ± 1.07	88.52 ± 2.06	0.03
Creatinine [mg/dl]	0.96 ± 0.02	1.11 ± 0.03	<0.001
eGFR [ml/min]	82.78 ± 1.51	79.76 ± 1.84	0.25
Uric acid [mg/dl]	7.25 ± 1.68	5.49 ± 0.22	0.18
hsCRP [mg/l]	1.77 ± 0.44	0.74 ± 0.18	0.06
Total cholesterol [mg/dl]	179.97 ± 6.16	190.52 ± 6.38	0.35
HDL [mg/dl]	54.25 ± 1.89	53.20 ± 1.87	0.89
LDL [mg/dl]	105.72 ± 5.62	115.96 ± 5.47	0.26
Triglycerides [mg/dl]	90.42 ± 7.97	106.84 ± 15.71	0.71
Sodium [mmol/l]	137.83 ± 0.27	138.16 ± 0.26	0.54
Potassium [mmol/l]	4.04 ± 0.04	4.18 ± 0.05	0.10

BMI: body mass index; WBC: white blood cells; RBC: red blood cells; MCV: mean (red blood) cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets; PDW: platelet distribution width; eGFR: estimated glomerular filtration rate; hsCRP: high-sensitivity C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

divided by the overall mean of all duplicated samples and then multiplied by 100, as previously described [19]. The intra-assay CV was less 6%, and interassay was less 10%.

2.7. Measurement of Prostanoids Levels. Plasma concentrations of TxB_2 and 6-ketoPGF-1alpha were determined using commercial immunoassays by Enzo Life Science, strictly following manufacturer's instructions, as previously described [19].

2.8. Statistical Analysis. Data is expressed as the mean ± SEM. The differences between two continuous parameters were assessed using the Mann–Whitney U test or Student t-test, following the Shapiro-Wilk test and Levene test as appropriate. Proteomics data analysis was performed as described in previous section.

3. Results

3.1. Baseline Characteristics of Investigated Population in Subgroups. The discriminating aggregation divided the study population into two groups. The aggregation was performed using arachidonic acid (1 mM working concentration) added to platelet-rich plasma in 5 minutes following its incubation with ASA at (30 μM, for 5 minutes). Following the agonist application, the reaction progress was recorded for 6 min. A complete inhibition of the aggregation by ASA was the criterion for distinguishing the AS from AR subjects. The first one constituted 36 subjects whose platelets were resistant to ASA (aspirin resistant (AR)). The second one (age and sex matched) was formed from 25 individuals that presented preserved ASA responsiveness (aspirin sensitive (AS)). The groups' characteristics are shown in Table 1. In a complete blood count, the differences between groups involved only quantitative parameters of red blood cells, hemoglobin level, hematocrit and red blood cell count that were significantly higher in AS. There were no differences observed in the qualitative measurements of erythrocyte line (MCV, MCH, and MCHC).

Regarding biochemical risk factors, significant differences (AR versus AS) concerned only fasting glucose (83.39 ± 1.07 versus 88.52 ± 2.06 mg%, resp., $p < 0.05$) and serum creatinine (0.96 ± 0.02 versus 1.11 ± 0.03 mg%, resp., $p < 0.05$). However, these values were maintained within normal ranges.

3.2. Platelet Function. There were no significant differences in platelet count (PLT), size (PDW), and activation level

Table 2: Comparison of prostanoids and markers of platelet activation in subgroups separated according to ASA response.

—	Decreased ASA response (mean ± SEM)	Normal ASA response (mean ± SEM)	p
N	36–	25–	—
PAI-1 [ng/ml]	3.11 ± 0.31	3.66 ± 0.32	0.04
Sel-P [ng/ml]	37.45 ± 2.10	40.26 ± 3.07	0.56
TxB_2 [pg/ml]	1557.8 ± 410.7	826.2 ± 144.6	0.28
6-keto-PGF1α [pg/ml]	215.16 ± 20.77	187.98 ± 18.41	0.40

PAI-1: plasminogen activator inhibitor-1; Sel-P: P-selectin; TxB_2: thromboxane B_2, 6-keto-PGF1α: 6-keto prostaglandin F1α.

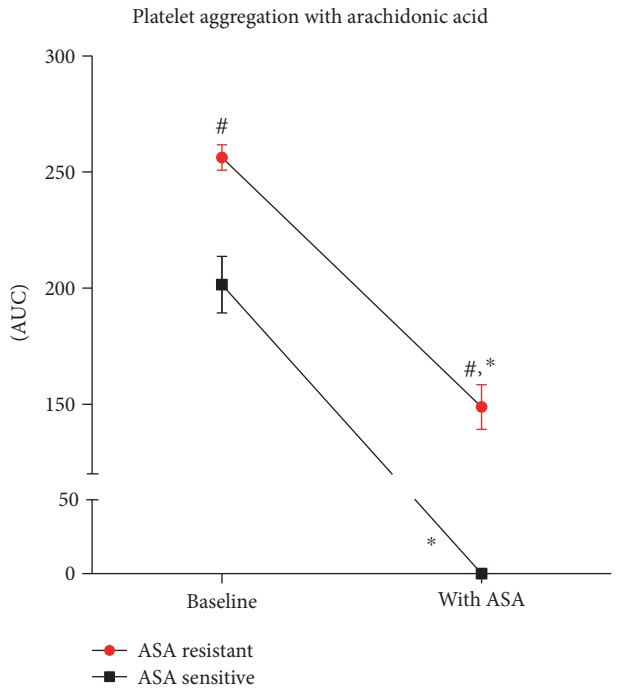

Figure 2: Platelet aggregation in response to arachidonic acid. Aggregations performed in platelet-rich plasma at baseline and after preincubation with ASA; AUC: area under curve of aggregation; $^{*}p < 0.001$ versus baseline; $^{\#}p < 0.001$ versus the control group.

(sP-selectin concentration) between subgroups. Nevertheless, AR subjects were characterized by lower plasminogen activator inhibitor-1 (PAI-1) concentrations (3.1 ± 0.3 versus 3.66 ± 0.3 ng/ml in AS, resp., $p < 0.05$; Table 2).

The AS population presented no platelet aggregation in response to arachidonic acid after PRP was preincubated with a fixed dose of aspirin. Otherwise, in AR, the aggregation was only partially blocked (256.2 ± 5.2 versus 148.8 ± 9.3 AU, $p < 0.001$). Furthermore, the difference between study groups was observed not only after ASA incubation (148.8 ± 9.5 versus 0.08 ± 0.08 AU, $p < 0.001$) but also at baseline (AA-induced aggregation without ASA, 256.2 ± 5.2 versus 201.4 ± 12.0 AU, $p < 0.001$) (Figure 2).

3.3. Proteomic Analysis. After preliminary analytical procedures and focusing on statistically significant proteins, we identified an average of 842 proteins per patient, based on an average of 7733 peptides. The obtained results demonstrated the reproducible sample preparation, enabling thorough quantitative analysis. In the next step, the identified proteins were divided according to molecular functions and their involvement in biological processes as shown in Figures 3 and 4. Subsequent quantitative analysis revealed differentiating proteins. The differential analysis of platelet proteome between study groups showed that the only discriminatory protein, affecting the response to aspirin, is carbonic anhydrase II. Exactly the same results were obtained in different settings, both in the collagen-induced aggregates and in the nonaggregated platelets (Table 3).

4. Discussion

This is the first study to demonstrate that carbonic anhydrase II (CA II) may be linked to human aspirin resistance (AR). The CA II protein (enzyme catalogue (EC) 4.2.1.1) in platelets has been described for the first time over 30 years ago [20] and first mentioned 60 years ago [21]. It catalyzes H^+ and HCO_3^- generation from CO_2 and H_2O contributing to pH changes [22]. Nevertheless, the exact role of CA II in the platelet pathophysiology remains not fully understood. A mutation in the CA II gene results in morphological and functional changes in mouse platelets [23].

The groups in our study were homogenous allowing to assume that the only discriminator is aspirin response. We used the LC/MS technique, which is much more reliable and investigator independent than the other ones, including the 2-dimensional electrophoresis [24, 25].

One of the explanation of the CA II, ASA interaction, observed in this study may be a hypothesis that by adjusting of the cytosolic pH [22], the CA II affects the acetylating of platelet cyclooxygenase by aspirin which could in turn determine their response to the ASA.

We have shown that platelets with greater CA II concentrations not only require lower arachidonic acid concentration to overcome ASA blockage but also produce more potent baseline aggregation. We postulate that platelet CA II increases platelet sensitivity to agonists like adrenaline, thrombin, and arachidonic acid. For this reason, humans with higher platelet CA II concentration and/or activity might be at higher risk of thromboembolic events. Hence, we postulate that the importance of CA in cardiovascular medicine seems to be still underestimated. Interestingly, numerous drugs commonly used in cardiovascular medicine (mainly diuretics) inhibit CA. The CA II activity can be stimulated by adrenaline [26]. Furthermore, adrenaline's potential to initiate aggregation is proportional to the platelet CA II activity and may by attenuated by the CA inhibitors like chlorthalidone [27]. Acetazolamide, a selective CA II inhibitor, decreases both basal- and adrenaline-induced platelet cytosolic chloride concentration and decreases thrombin sensitivity [28] similarly to another CA inhibitor (ethoxzolamide) [29]. Such effect may be also obtained by eliminating CO_2 from the platelet environment [30].

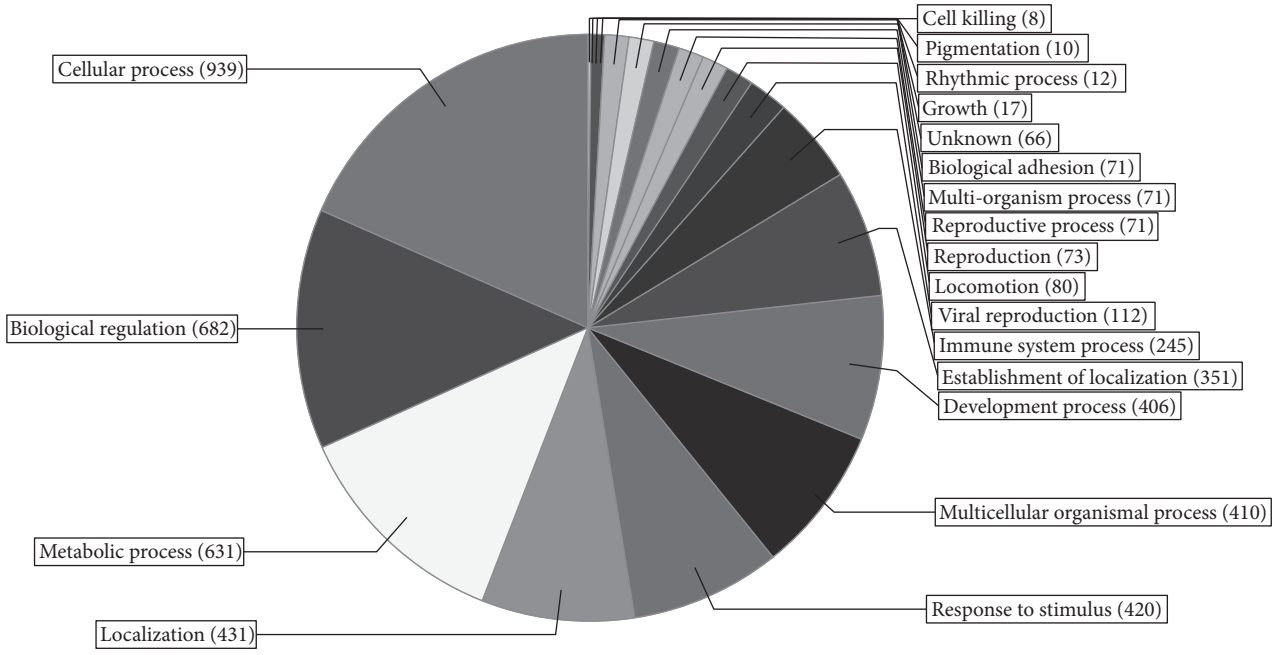

Figure 3: Platelet proteome organized according to involvement in biological processes of recognized proteins.

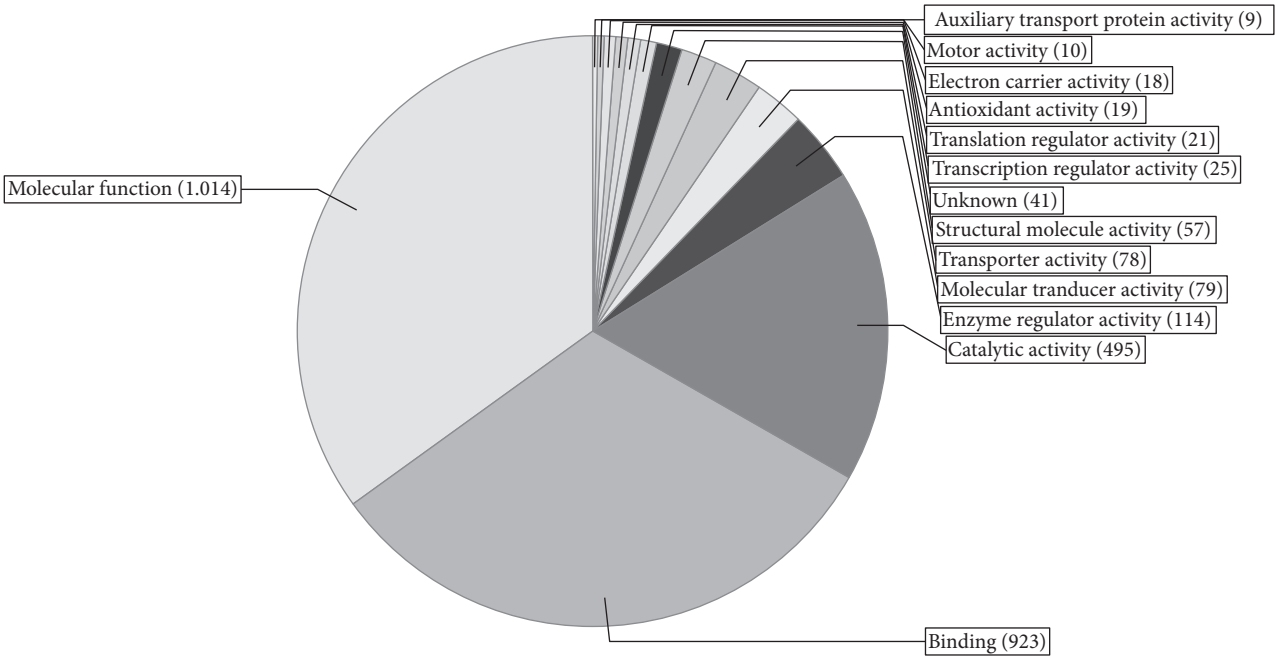

Figure 4: Platelet proteome organized according to molecular function of recognized proteins.

Table 3: Quantitative LC/MS analysis of proteomes of platelets sensitive versus resistant to ASA (only discriminatory proteins are specified).

Material	Protein	q value	Ratio	Fold change	Number of peptides	Protein name
Collagen-induced aggregation pellets	P00918	0.00213	0.67	1.49	18	*Carbonic anhydrase 2 OS = Homo sapiens*
Inactivated platelets	P00918	0.00106	0.69	1.44	19	*Carbonic anhydrase 2 OS = Homo sapiens*

What is more, the authors of a recent systemic review confirmed that thiazide-like diuretics have a superiority in reducing cardiovascular events over thiazide-type ones independently of lowering the blood pressure [31], which might be due to their activity against CA II and subsequent platelet aggregation [27].

Our results regarding sex distribution among AR population confirm the data published by other groups [32], and it should be verified if platelet CA II presents different sex-dependent activities. Lower red blood cells (RBC), hematocrite, and the hemoglobin levels in the AR population are accompanied by higher platelet CA II content. Anemia caused by iron deficiency has been demonstrated to be a risk factor for ischemic stroke due to reactive thrombocytosis, which might be a compensatory mechanism providing sufficient CA activity, normally substantially maintained by RBCs [33]. Platelet CA II might cooperate with the CA in RBCs in maintaining the acid-based blood balance. In our study, a thrombocytosis was not well marked, but increased CA II expression might induce differences in platelet function.

5. Conclusions

Carbonic anhydrase II (CA II) is a modulator of platelet function. Increased activity and/or concentration of CA II in platelets should be rated as a new independent risk factor for aspirin resistance and thus for thromboembolic events. There may be a need to use the drugs inhibiting CA in clinical setting more often nowadays, especially in patients with increased platelet activity/amount of CA II.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Research Article

Increased Levels of Oxidative Stress Markers, Soluble CD40 Ligand, and Carotid Intima-Media Thickness Reflect Acceleration of Atherosclerosis in Male Patients with Ankylosing Spondylitis in Active Phase and without the Classical Cardiovascular Risk Factors

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Objective. The primary aim of the study was to assess levels of oxidative stress markers, soluble CD40 ligand (sCD40L), serum pregnancy-associated plasma protein-A (PAPP-A), and placental growth factor (PlGF) as well as carotid intima-media thickness (IMT) in patients with ankylosing spondylitis (AS) with active phase without concomitant classical cardiovascular risk factors. *Material and methods*. The observational study involved 96 male subjects: 48 AS patients and 48 healthy ones, who did not differ significantly regarding age, BMI, comorbid disorders, and distribution of classical cardiovascular risk factors. In both groups, we estimated levels of oxidative stress markers, lipid profile, and inflammation parameters as well as sCD40L, serum PAPP-A, and PlGF. In addition, we estimated carotid IMT in each subject. *Results*. The study showed that markers of oxidative stress, lipid profile, and inflammation, as well as sCD40L, PlGF, and IMT, were significantly higher in the AS group compared to the healthy group. *Conclusion*. Our results demonstrate that ankylosing spondylitis may be associated with increased risk for atherosclerosis.

1. Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory arthritis affecting primarily the axial skeleton and sacroiliac joints [1, 2].

Several epidemiological studies confirmed the high risk of cardiovascular morbidity and mortality in AS patients, which is associated with an increase of risk for atherosclerosis independent of traditional risk factors that may be connected with the disease activity, functional and mobility limitations, structural damage, and inflammation [3, 4].

Inflammation, on the one hand, has an important role in different stages of atherogenesis, and, on the other, attenuates established cardiovascular risk factors [5, 6].

During inflammation and phagocytosis reactions, reactive oxygen species (ROS) may be released into the extracellular space and injure the surrounding tissue and thereby result in the production of acute-phase proteins [7].

Moreover, according to the theory of oxidative stress, atherosclerosis results from the oxidative modification of low-density lipoproteins (LDL) in the arterial wall by ROS [8]. Modified ox-LDL is generally accepted to be an important elicitor of promitotic, proinflammatory, and atherogenic effects in vascular cells [9]. What is more, ox-LDL may interact with different molecules and form proatherogenic complexes (e.g., ox-LDL/CRP and ox-LDL/β2-glycoprotein 1) that may not only perpetuate vascular inflammation but also trigger autoimmune responses, accelerating the development of atherosclerosis [10, 11].

In addition, high titers of antibodies of ox-LDL have been reported in patients with myocardial infarction [12], atheroslerosis [13], and rheumatoid disease [9].

It has been demonstrated that the CD40L concentration is increased in patients with occlusive carotid artery disease and may be also a predictor of cardiovascular events [14, 15].

On the other hand, it has been also reported that the placental growth factor (PlGF) plays an important role in atherosclerosis by stimulating the angiogenesis and atherogenic migration of monocytes/macrophages into the arterial wall [16]. It seems to be more effective during the early phase of atherogenesis, because anti-PlGF antibody treatment significantly inhibits early lesions but is ineffective during the more advanced stages of plaque development [17].

The other marker of atherogenesis is serum pregnancy-associated plasma protein-A (PAPP-A). It has been shown that its circulating concentrations are higher in patients with acute coronary syndrome than in patients with chronic stable angina and in healthy subjects. Furthermore, increased serum PAPP-A concentration is associated with the presence and extent of stable coronary heart disease as well as predictive of future ischemic cardiac events, and the need for percutaneous coronary intervention or coronary artery bypass graft surgery [18, 19].

On the other hand, a few papers postulate that oxidative stress might be involved both in AS disease onset and progression [20–24].

Taking this data into account, the primary aim of the study was to assess levels of oxidative stress markers, sCD40L, PAPP-A, and PlGF as well as carotid IMT in AS patients with active phase without concomitant classical cardiovascular risk factors.

2. Materials and Methods

2.1. Participants. The research protocol has been reviewed and approved by the Bioethical Committee of the Medical University of Silesia in Katowice (Permission number KNW/022/KB/103/16), and all the subjects we analyzed gave their informed, written consent for inclusion in the observational study. It was carried out in accordance with the Declaration of Helsinki (1964). The study involved 96 male subjects: 48 patients with ankylosing spondylitis (AS group, mean age 46.06 ± 1.44 years) and 48 healthy subjects (control group, mean age 46.63 ± 1.50 years), who did not differ significantly regarding age, BMI, comorbiding disorders, and distribution of classical cardiovascular risk factors. All the patients included in the observational study fulfilled the modified New York Criteria for definite diagnosis of AS, which served as the basis for the ASAS/EULAR recommendations [25]. Enrollment in the study was performed in the AS group of male patients, with definite diagnosis of AS who did not suffer from any other diseases, had no associated pathologies, and whose attending physician did not apply disease-modifying antirheumatic drugs (DMARDs), biologic agents, or steroids. The AS patients were treated with NSAIDs, which doses were not altered within one month before the beginning of the study. All the patients with AS were HLAB27 positive, and they exhibited III and IV radiographic grades of sacroiliac joint disease. The BASDAI was 5.35 ± 1.64 and the BASFI was 5.13 ± 2.17. The AS patients did not suffer from any other diseases. Similarly, the healthy subjects had no acute or chronic diseases, nor did they use any medication. The demographic data of the subjects is shown in Table 1.

Table 1: Demographic data of the study subjects.

Characteristic	AS patients ($n = 48$)	Healthy subjects ($n = 48$)	p value
Sex (M/F)	48/0	48/0	—
Age (years), mean (SD)	46.06 ± 1.44	46.63 ± 1.50	0.096
BMI (kg/m^2), mean (SD)	24.5 ± 4.4	23.8 ± 5.7	0.674
Smoking (yes/no)	0/48	0/48	—
BASDAI	5.35 ± 1.64	—	—
BASFI	5.13 ± 2.17	—	—
HLA B27 antigen (yes/no)	48/0	—	—
Cerebral/coronary/peripheral vascular disease (yes/no)	0/48	0/48	—
Hypertension (yes/no)	0/48	0/48	—
Diabetes mellitus (yes/no)	0/48	0/48	—
Hyperlipidemia (yes/no)	0/48	0/48	—
Medication			
NSAID (yes/no)	48/0	—	—
DMARD (yes/no)	0/48	—	—
Biological agents (yes/no)	0/48	—	—

SD: standard deviation; BMI: body mass index; BASDAI: the Bath Ankylosing Spondylitis Disease Activity Index; BASFI: the Bath Ankylosing Spondylitis Functional Index; HLA B27 antigen: human leukocyte B27 antigen; NSAID: nonsteroidal anti-inflammatory drug; DMARD: disease-modifying antirheumatic drug.

The subjects from both groups were asked to abstain from alcohol, drugs, and any immunomodulators, immunostimulators, hormones, vitamins, minerals, or other substances with antioxidant properties for 4 weeks before the study. All the subjects were also asked to refrain from the consumption of caffeine 12 hours prior to laboratory analysis.

2.2. Blood Sample Collection. Blood samples of all the subjects were collected in the morning before the first meal. Samples of whole blood (5 ml) were drawn from a basilic vein of each subject and then collected into tubes containing ethylenediaminetetraacetic acid (Sarstedt, S-monovette with 1.6 mg/ml EDTA-K_3, catalogue number 04.1931) and into tubes with a clot activator (Sarstedt, S-monovette, catalogue number

04.1934). The blood samples were centrifuged (10 min., 900 *g*, 4°C), and then the plasma and serum were immediately separated and stored at the temperature of −70°C, until biochemical analyses were performed. In turn, the red blood cells retained from removal of EDTA-plasma were rinsed with isotonic salt solution and then 10% of hemolysates were prepared for further analyses. Hemoglobin concentration in hemolysates was determined by standard cyanmethemoglobin method. The inter- and intra-assay coefficients of variations (CV) were, respectively, 1.1% and 2.4%.

2.3. Biochemical Analyses

2.3.1. Oxidative Stress Marker Analyses

(1) Determination of Activity of Antioxidant Enzymes. The plasma and erythrocyte superoxide dismutase (SOD—E.C.1.15.1.1) activity was determined by the Oyanagui method [26]. Enzymatic activity was expressed in nitrite unit (NU) in each mg of hemoglobin (Hb) or ml of blood plasma. One nitrite unit (1 NU) means a 50% inhibition of nitrite ion production by SOD in this method. SOD isoenzymes (SOD-Mn and SOD-ZnCu) were measured using potassium cyanide as the inhibitor of the SOD-ZnCu isoenzyme. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.8% and 5.4%.

The catalase (CAT—E.C.1.11.1.6.) activity in erythrocytes was measured by Aebi [27] kinetic method and expressed in IU/mgHb. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.6% and 6.1%.

The erythrocyte glutathione peroxidase (GPx—E.C.1.11.1.9.) activity was assayed by Paglia and Valentine's kinetic method [28], with t-butyl peroxide as a substrate and expressed as micromoles of NADPH oxidized per minute and normalized to one gram of hemoglobin (IU/gHb). The inter- and intra-assay coefficients of variations (CV) were, respectively, 3.4% and 7.5%.

The activity of glutathione reductase in erytrocytes (GR—E.C.1.6.4.2) was assayed by Richterich's kinetic method [29], expressed as micromoles of NADPH utilized per minute and normalized to one gram of hemoglobin (IU/gHb). The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.1% and 5.8%.

(2) Determination of Nonenzymatic Antioxidant Status. The total antioxidant capacity of plasma was measured as the ferric reducing ability of plasma (FRAP) according to Benzie and Strain [30] and calibrated with the use of Trolox and expressed in μmol/l. The inter- and intra-assay coefficients of variations (CV) were, respectively, 1.1% and 3.8%.

The serum concentration of protein sulfhydryl (PSH) was determined by Koster et. al's method [31] using dithionitrobenzoic acid (DTNB) and expressed in μmol/l. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.6% and 5.4%.

The serum concentration of uric acid (UA) was determined by a uricase-peroxidase method [32] on the Cobas Integra 400 plus analyzer and expressed as mg/dl. The inter- and intra-assay coefficients of variations (CV) were, respectively, 1.4% and 4.4%.

(3) Determination of Lipid Peroxidation Products and TOS. The intensity of lipid peroxidation in the plasma and the erythrocytes was measured spectrofluorimetrically as a thiobarbituric acid-reactive substances (TBARS) according to Ohkawa et al. [33]. The TBARS concentrations were expressed as malondialdehyde (MDA) equivalents in μmol/l in plasma or nmol/gHb in erythrocytes. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.1% and 8.3%.

The serum concentrations of oxidized low-density lipoprotein (ox-LDL) and antibodies to ox-LDL (ab-ox-LDL) were measured with the use of ELISA kits (catalogue numbers BI-20022 and BI-20032, Biomedica, Poland) according to the manufacturer's instructions. The ox-LDL and the ab-ox-LDL concentrations were expressed in ng/ml and mU/ml, respectively. The inter- and intra-assay coefficients of variations (CV) for ox-LDL were 5.8% and 9.4%, respectively, and for ab-ox-LDL—4.1% and 8.7%, respectively.

The serum total oxidant status (TOS) was determined with the method described by Erel [34] and expressed in μmol/l. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.2% and 6.4%.

2.3.2. Determination of Inflammatory State Parameters. The erythrocyte sedimentation rate (ESR) was determined immediately in whole blood with EDTA by the classical Westergren method.

The high-sensitivity C-reactive protein (hs-CRP) (catalogue number EIA 4584) concentration in serum was determined by latex immunoturbidimetric method (BioSystems, Spain) and expressed in mg/l. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.3% and 5.5%.

The serum ceruloplasmin (CER) oxidase activity was measured with the use of the p-phenylenediamine kinetic method by Richterich [29] and expressed in mg/dl after a calibration with pure ceruloplasmin isolated from a healthy donor serum pool. The inter- and intra-assay coefficients of variations (CV) were, respectively, 3.1% and 6.1%.

2.3.3. Determination of Lipid Profile. Total, HDL-, and LDL-cholesterol (T-Chol, HDL-Chol, and LDL-Chol, resp.) and triglicerydes (TG) concentrations in serum were estimated using routine techniques (Cobas Integra 400 plus analyzer, Roche Diagnostics, Mannheim, Germany). The concentrations were expressed in mg/dl. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.8% and 5.4% for T-Chol, 3.2% and 5.4% for HDL-Chol, 2.6% and 6.5% for LDL-Chol, and 2.5% and 7.6% for TG.

The triglycerides/HDL-cholesterol (TG/HDL) and LDL-cholesterol/HDL-cholesterol (LDL/HDL) ratios were calculated.

2.3.4. Determination of PAPP-A, Soluble CD40 Ligand, and PlGF. Serum pregnancy-associated plasma protein-A (PAPP-A) (catalogue number EIA-4512), soluble CD40

Table 2: Parameters of enzymatic antioxidant status (superoxide dismutase (SOD), its isoenzymes: manganese superoxide dismutase (SOD-Mn) and copper-zinc superoxide dismutase (SOD-CuZn), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) activity) and nonenzymatic antioxidant status (ferric reducing ability of plasma (FRAP), protein sulfhydryl (PSH), and uric acid (UA) concentration, as well as activity of ceruloplasmin (CER)) in ankylosing spondylitis (AS) patients and healthy subjects.

Parameter	AS patients ($n = 48$)	Healthy subjects ($n = 48$)	p
SOD (p) (NU/ml)	12.67 ± 1.98	10.93 ± 2.55	**<0.001**
SOD-Mn (p) (NU/ml)	5.08 ± 2.00	4.59 ± 1.88	0.223
SOD-CuZn (p) (NU/ml)	7.64 ± 2.31	6.79 ± 1.99	0.055
SOD (e) (NU/mgHb)	105.85 ± 22.60	95.50 ± 19.18	**0.017**
CAT (e) (IU/mgHb)	410.98 ± 63.56	352.55 ± 77.21	**<0.001**
GPx (e) (IU/gHb)	27.23 ± 6.43	24.49 ± 5.00	**0.022**
GR (e) (IU/gHb)	1.67 ± 0.58	1.38 ± 0.43	**0.007**
FRAP (p) (μmol/l)	550.38 ± 76.98	642.17 ± 105.67	**<0.001**
PSH (s) (μmol/l)	474.46 ± 192.06	559.07 ± 215.14	**0.045**
UA (s) (mg/dl)	4.73 ± 1.39	5.84 ± 1.53	**<0.001**

Values are expressed as means ± standard deviations (SD) of the means; p: plasma; s: serum; e: erythrocyte lysates.

Ligand (sCD40L) (catalogue number EIA4851), and placental growth factor (PlGF) (catalogue number EIA-4529) concentrations were assayed by ELISA methods with DRG Instruments GmbH (Germany) kits. All assays were performed according to the manufacturer's instructions. The PAPP-A and sCD40L concentrations were expressed in ng/ml, the PlGF concentration—in pg/ml. The inter- and intra-assay coefficients of variations (CV) were, respectively, 6.8% and 10.2% for PAPPA-A, 5.1% and 9.4% for sCD40L, and 6.2% and 12.1% for PlGF.

2.4. Assay of Intima-Media Thickness. A high-resolution Doppler ultrasonography was performed with a Logic-5 device with a high-frequency (11 MHz and 15 MHz) linear probe. The sonographer was an angiologist who was unaware of subject's clinical state. Measurement of intima-media thickness (IMT) was performed in the right and left common carotid arteries, and the average of the 2 measurements was calculated. The IMT was expressed in mm.

2.5. Assay of Activity of Ankylosing Spondylitis. The activity of ankylosing spondylitis was measured by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the Bath Ankylosing Spondylitis Functional Index (BASFI).

The BASDAI has six questions related to fatigue, back pain, peripheral pain, peripheral swelling, local tenderness, and morning stiffness (degree and length). Other than the item relating to morning stiffness, all questions were scored from 0 (none) to 10 (very severe) using a visual analogue scale (VAS). The sum was calculated as the mean of two morning stiffness items and the four remaining items [35].

The BASFI is the mean score of ten questions addressing functional limitations and the level of physical activity at home and work, assessed on VAS scales (0 = easy, 10 = impossible) [36].

2.6. Statistical Analyses. Statistical analyses were undertaken using the statistical package of Statistica 10 Pl software. For each parameter, the indicators of the descriptive statistics were determined (mean value and standard deviation (SD)). The normality of the data distribution was checked using the Shapiro-Wilk test, while the homogeneity of the variance was checked by applying the Levene test. In order to compare the differences between the control group and the AS group, an independent sample Student's t-test was used or alternatively the Mann–Whitney U test. Correlations between particular parameters were statistically verified by means of Spearman's nonparametric correlation test. Differences at the significant level of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Oxidative Stress. In AS patients, there was a significantly higher activity of antioxidant enzymes: plasma SOD, along with erythrocyte SOD, erythrocyte CAT, erythrocyte GPx, and erythrocyte GR, was observed in comparison to the healthy subjects. But the plasma activity of SOD-Mn and SOD-CuZn isoenzymes in both groups did not differ significantly. What is more, in AS patients, a significantly lower concentration of the parameters of nonenzymatic antioxidants, FRAP, PSH, and UA, was observed in comparison to the healthy subjects (Table 2).

Furthermore, a significantly higher concentration of lipid peroxidation products plasma MDA, along with erythrocyte MDA, ox-LDL, and ab-ox-LDL, was noted in AS patients in comparison to the control group of the healthy subjects. In addition, in AS patients, the concentration of TOS was significantly higher in comparison to the control group (Table 3).

3.2. Lipid Profile, Inflammatory Parameters, and Carotid Intima-Media Thickness. In AS patients, there was a significantly higher concentration of the lipid profile parameters: T-Chol, LDL-Chol, and TG as well as TG/HDL ratio and LDL/HDL ratio were noted in comparison to the control group. But only the concentration of HDL-Chol in both groups did not differ significantly. What is more, also the concentrations of sCD40L and PlGF as well as carotid IMT

Table 3: Oxidative stress parameters: malondialdehyde (MDA), oxidized low-density lipoprotein (ox-LDL), antibodies to oxidized low-density lipoprotein (ab-ox-LDL), and total oxidant status (TOS) concentration in ankylosing spondylitis (AS) patients and healthy subjects.

Parameter	AS patients ($n = 48$)	Healthy subjects ($n = 48$)	p
MDA (p) (μmol/l)	2.51 ± 0.63	2.25 ± 0.47	**0.025**
MDA (e) (nmol/gHb)	0.17 ± 0.03	0.15 ± 0.03	**<0.001**
ox-LDL (s) (ng/ml)	268.48 ± 105.83	162.98 ± 63.29	**<0.001**
ab-ox-LDL (s) (mU/ml)	479.82 ± 328.39	323.82 ± 210.26	**0.007**
TOS (s) (μmol/l)	26.99 ± 10.65	16.50 ± 6.87	**<0.001**

Values are expressed as means ± standard deviations (SD) of the means; p: plasma; s: serum; e: erythrocyte lysates.

were significantly higher in AS patients in comparison to the healthy group. The concentration of PAPP-A in both groups did not differ significantly. Furthermore, in AS patients, a significantly higher concentration of all the examined parameters of inflammatory state, ESR, hs-CRP, and CER, was observed in comparison to the healthy subjects (Table 4).

3.3. Significant Relationships among the Estimated Parameters in AS Patients. In the AS group, a high, statistically significant correlation was observed between acute phase proteins (hs-CRP versus CER; $r = 0.59$, $p < 0.05$), total SOD activity in erythrocytes and ceruloplasmin ($r = 0.52$, $p < 0.05$), and glutathione cycle enzyme activities in erythrocytes (POX versus GR; $r = 0.66$, $p < 0.05$) as well as between uric acid concentration and FRAP activity in plasma ($r = 0.65$, $p < 0.05$). Mild but still statistically significant correlations were shown between CRP concentration and SOD-CuZn and SOD-Mn plasma activities ($r = -0.35$, $p < 0.05$), erythrocyte MDA concentration and SOD erythrocyte activity ($r = 0.35$, $p < 0.05$), sCD40L and PlGF concentration ($r = 0.51$, $p < 0.05$), plasma MDA and T-Chol ($r = 0.30$, $p < 0.05$), and TOS and TG/HDL ratio ($r = 0.37$, $p = 0.001$). Also, a high correlation between BASFI and BASDAI was observed ($r = 0.67$, $p < 0.05$) in AS patients.

Unfortunately, no statistically significant correlations between AS activity parameters (BASFI and BASDAI) and oxidative stress parameters as well as carotid IMT and oxidative stress parameters were obtained.

Table 4: Parameters of lipid profile (total cholesterol (T-Chol), low-density lipoprotein cholesterol (LDL-Chol), high-density lipoprotein cholesterol (HDL-Chol), triglicerydes (TG) concentration, TG/HDL, and LDL/HDL ratio), concentration of PAPP-A, soluble CD40 ligand (sCD40L), PlGF, and value of carotid intima-media thickness (IMT), as well as parameters of inflammatory state (erythrocyte sedimentation rate (ESR) value, high sensitivity C-reactive protein (hs-CRP), and ceruloplasmin (CER) concentration) in ankylosing spondylitis (AS) patients and healthy subjects.

Parameter	AS patients ($n = 48$)	Healthy subjects ($n = 48$)	p
T-Chol (s) (mg/dl)	217.73 ± 35.48	187.09 ± 18.57	**<0.001**
LDL-Chol (s) (mg/dl)	140.49 ± 33.64	112.57 ± 22.89	**<0.001**
HDL-Chol (s) (mg/dl)	61.10 ± 18.08	57.49 ± 15.16	0.291
TG (s) (mg/dl)	190.48 ± 47.30	139.74 ± 47.66	**<0.001**
TG/HDL ratio	3.37 ± 1.13	2.57 ± 1.23	**<0.001**
LDL/HDL ratio	2.55 ± 1.1	2.02 ± 0.58	**<0.05**
PAPP-A (s) (ng/ml)	17.82 ± 16.22	14.24 ± 4.35	0.281
sCD40L (s) (ng/ml)	8.93 ± 3.74	5.54 ± 2.37	**<0.001**
PlGF (s) (pg/ml)	25.8 ± 8.99	19.77 ± 3.27	**<0.001**
Carotid IMT (mm)	1.1 ± 0.13	0.55 ± 0.08	**<0.001**
ESR	27.13 ± 21.55	5.94 ± 3.91	**<0.01**
hs-CRP (s) (mg/l)	14.92 ± 15.55	1.58 ± 2.00	**<0.001**
CER (s) (mg/dl)	48.12 ± 12.67	38.68 ± 4.84	**<0.001**

Values are expressed as means ± standard deviations (SD) of the means; s: serum.

4. Discussion

Briefly, in this observational study, we viewed significantly higher oxidative stress parameters, levels of inflammatory state, and lipid profile parameters, sCD40L, and PlGF as well as values of TG/HDL, LDL/HDL ratio, and carotid IMT in AS patients with active phase (BASDAI and BASFI), compared to the healthy subjects.

In the available literature, only a few, unequivocal reports concerning the prooxidant-antioxidant status in patients with ankylosing spondylitis have been published.

In the study [20], in patients with ankylosing spondylitis, a significantly lower plasma total antioxidant status (TAS) was demonstrated, as well as higher values of the total oxidant status (TOS) and oxidative stress index (OSI), in comparison to the control group of healthy volunteers. That study did not reveal any significant correlation between the values of the above parameters and the activity of the disease process.

In another study [22], no significant differences were demonstrated to occur in the activity of SOD, nitric oxide (NO) metabolites, and the concentration of MDA, between the group of patients with AS in the active form and the group of patients with inactive process. The activity of SOD, NO metabolites, and the concentration of MDA also failed to demonstrate statistically significant differences when compared to the control group of healthy subjects, whereas the activity of CAT and the concentration of MDA in patients with an active form of the disease were significantly higher, in relation to other groups of subjects studied.

In the study [37], all antioxidant enzyme activities were lower, but the MDA level was higher in patients with AS when compared to the control group.

The next paper [38] reported that ESR, CRP, and lipid peroxidation products were higher in patients with AS than in healthy subjects, but vitamins A, C, E, and β-carotene concentrations in plasma, reduced glutathione, and glutathione peroxidase activity values in erythrocyte were lower in patients with AS than in healthy subjects. But the authors estimated only some chosen parameters of prooxidant-antioxidant status. Furthermore, the level of oxidative stress was shown to be correlated with the intensity of inflammation in patients with AS [21].

Contrastingly, there are many papers, which reported increased cardiovascular risk in AS patients [39–43].

In the current study, a significant increase in acute-phase protein concentration (hs-CRP and CER) was observed in AS patients compared to the healthy subjects. A significant positive correlation between hs-CRP and CER was shown as well as a significant negative correlation between hs-CRP and plasma SOD-CuZn.

Some researchers found that increased AS disease activity was associated with decreases in lipid levels and the decrease in HDL-Chol levels, which tended to be almost twice as large as the decrease in total cholesterol levels, resulting in a more atherogenic lipid profile [43, 44].

However, in the study [45] in AS patients, the authors did not observe a significant difference in T-Chol, LDL-Chol, HDL-Chol, and TG concentration compared to healthy subjects. But in AS patients, the values of HDL/LDL ratio, complex intima-media, and TOS concentration as well as inflammatory state parameters were significantly higher than in controls. In this study, a significant increase in T-Chol, LDL-Chol, and TG as well as TG/HDL and LDL/HDL ratio was observed in comparison to the healthy subjects. But HDL-Chol concentration did not differ between AS patients and healthy subjects. In addition, the carotid IMT in AS patients was also significantly higher in comparison to the healthy subjects.

A high level of cholesterol, especially in LDL, may activate thrombocytes and cause the release of substances that activate phospholipase A_2. Then, the accumulated arachidonic acid is metabolized to leukotriene by a lipoxygenase pathway and thromboxane, prostaglandin, and MDA by a cyclooxygenase pathway. During this metabolism, ROS may be produced, and under insufficient antioxidant capacity, they may also trigger lipid peroxidation [44]. What is more, it was shown that the TG/HDL ratio estimates atherogenic small, dense low-density lipoprotein cholesterol and predicts arterial stiffness and hard cardiovascular events in adults [46, 47]. In our study, we observed a significant positive correlation between TOS and TG/HDL ratio.

Additionally, it was also shown that an increase in serum LDL levels leads to an increase in the adherence of circulating monocytes to arterial endothelial cells and at the same time to an increased rate of entry of LDL into the intima [48]. It is also possible that TG enrichment may alter the physicochemical properties of LDL, which is considerably more susceptible to oxidation [49].

In this study, we also observed the increased concentration of lipid peroxidation products in plasma and erythrocyte.

MDA is one the most abundant aldehydes, resulting from peroxidation of arachidonic, eicosapentaenoic, and docosahexaenoic acid [50, 51]. MDA reacts with lysine residues by forming Schiff bases [52] and plays a major role in LDL modification and their deviation towards macrophages [48].

In the current study, a significantly higher concentration of MDA in plasma as well as in erythrocytes in AS patients was observed compared to healthy subjects. A positive correlation between plasma MDA and T-Chol as well as erythrocyte MDA and SOD in AS patients was also shown. We did not observe any correlation between MDA and other estimated parameters. The explanation of this fact may be that TBARS assay does not measure MDA exclusively, because it reacts to compounds other than MDA [51].

However, so far, there are no reports estimating ox-LDL and ab-ox-LDL concentration in ankylosing spondylitis. In the present study, a significantly higher concentration of ox-LDL as well as ab ox-LDL in AS patients was observed compared to healthy subjects.

In the study [53], it was shown that antibodies to ox-LDL were correlated significantly with ESR and CRP in patients with early rheumatoid arthritis and suggested that the occurrence of these antibodies must be related to inflammation. However, in the current study, no correlation was observed between ox-LDL and ab-ox-LDL and other estimated parameters.

In our study, we observed a significantly higher levels of sCD40L and PlGF as well as a positive correlation between them. Furthermore, in this study, the level of PAPP-A did not differ between AS patients and healthy subjects. It may be connected with the fact that PAPP-A is expressed in unstable but not in stable atherosclerotic plaques [18].

The study proved that increased oxidative stress, the levels of sCD40L and PlGF, the disturbance of lipids, and the inflammation process may enhance atherogenesis in AS patients.

However, the study has some limitations. First, it involved only 48 AS patients and thus a greater number of patients should be examined. Second, patients in different stages of AS should be involved in the study.

5. General Conclusion

Our results demonstrate that increased oxidative stress, higher serum concentrations of PlGF and sCD40L, and increased IMT may reflect the acceleration of atherosclerosis in male AS patients in active phase and without concomitant classical cardiovascular risk factors.

Conflicts of Interest

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

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