OXIDATIVE STRESS REVISITED - MAJOR ROLE IN VASCULAR DISEASES

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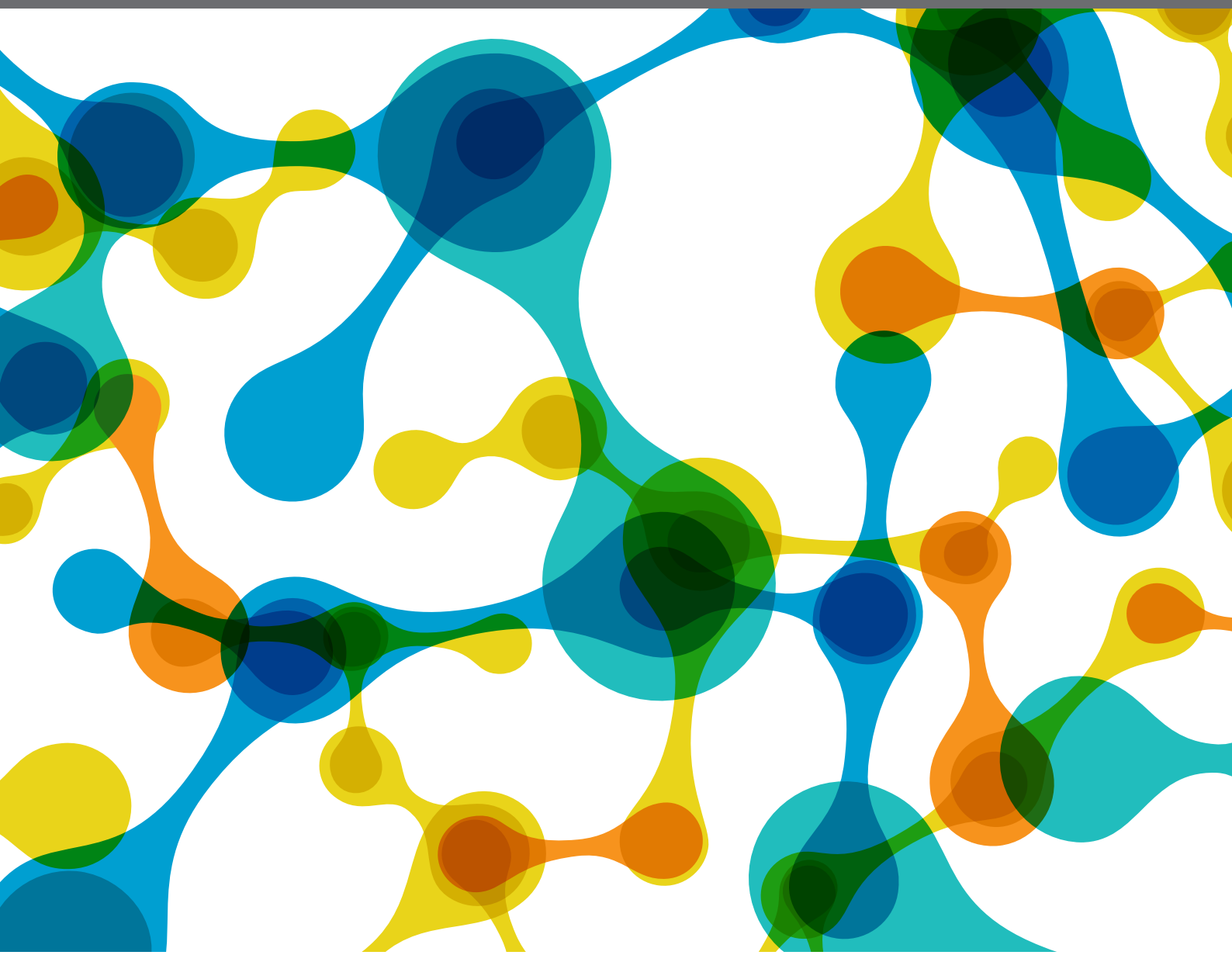

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OXIDATIVE STRESS REVISITED - MAJOR ROLE IN VASCULAR DISEASES

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Oxidative stress is an underlying factor in health and disease. Reactive oxygen species are produced as a result of normal cellular metabolism. The subsequent altered redox state between the formation and the neutralization of pro-oxidants results in their increased levels and therefore leads to cellular damage. Different research disciplines have increased our knowledge of the importance of this cell redox status and the recognition of oxidative stress as a process with implications for many pathophysiological states. Genetic and environmental factors, nutrition and lifestyle may indicate a pro-oxidative and pro-inflammatory state, linked to alterations in cellular structure and function. Oxidative stress emerges as a common, unifying factor in several conditions including diabetes and cardiovascular diseases.

This eBook aims to provide novel data regarding the role played by oxidative stress and inflammation in the development of chronic diseases and the different classes of therapeutics from the bench to the clinic, stressing the awareness of these concepts for the treatment of disease. In addition, articles addressing an overview of the role of oxidative stress in vascular diseases reviewing some current concepts indicating that oxidative stress and inflammation are key mechanisms linking vascular diseases and current state-of-the-art approaches to monitor, prevent and inhibit oxidative stress will be highlighted.

There is a close relation between oxidative stress, inflammation and cardiovascular diseases. Despite the great amount of investigation carried out in the field, there are still uncertainties about the mechanisms by which free radicals can modify tissues such as perivascular adipose tissue that ultimately will reflect on vascular function. This eBook will focus on articles that can explore and identify these mechanisms.

Concurrent with this understanding of oxidative stress milieu, it is necessary to recognize the need for new pharmacological tools effective in restoring oxidative balance. The abundance of new information and the paradigm shift in our understanding of how antioxidants and other redox-active drugs work in a wide variety of vascular diseases will be specifically highlighted. This eBook will provide a comprehensive, up-to-date source of information on the design and mechanistic, pharmacological, and medicinal aspects of redox-active therapeutics. Finally, a unique feature of the eBook is to provide a way to foster an enthralling discussion revisiting old paradigms and finding new solutions for the treatment of vascular diseases. The topic will include original research articles, hypotheses, perspectives and (mini)reviews from experts in the field.

The next decade shows promise for the translation of this body of knowledge to novel human therapeutics and this eBook will enable to increment our knowledge in this field.

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Table of Contents

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EDITORIAL
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Editorial: Oxidative Stress Revisited—Major Role in Vascular Diseases

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Keywords: oxidative stress, inflammation, aging, non-communicable diseases, novel therapeutic approaches

Editorial on the Research Topic

Oxidative Stress Revisited—Major Role in Vascular Diseases

Oxidative stress is an underlying factor in health and disease (Sies, 1985). The concept of oxidative stress was formulated in 1956 by Harman, and throughout the years substantial knowledge established that oxidative stress, an imbalance between oxidants and antioxidants with a disruption of redox signaling and control, is crucial for vascular diseases (Harman, 1956).

In this Frontiers Research topic oxidative stress was revisited by discussing old paradigms and suggesting new therapeutic approaches to decreased oxidative stress and reduce the progression of vascular diseases.

In the vasculature, several sources promote an increment in oxidative stress. Free radicals (such as the superoxide anion radical, hydrogen peroxide, hydroxyl radical, as well as the nitric oxide radical and peroxynitrite) are major sources of oxidative stress (Sies, 1985). Vascular oxidative stress promotes endothelial dysfunction and atherosclerosis progression (Sena et al.) playing a major role in several vascular diseases including neurodegenerative diseases (Carvalho and Moreira) and diabetic complications (Santiago et al.; Sena et al.; Teodoro et al). This was elegantly discussed by several authors in this research topic.

Oxidative stress may have a critical role in the neurovascular uncoupling underlying brain aging and dysfunction. Carvalho and Moreira discussed how oxidative stress is in the front line of vascular alterations observed in brain aging and neurodegenerative conditions, particularly Alzheimer disease.

In addition, Lourenço et al. demonstrated that non-pathological brain aging involves changes in both neurovascular and neurometabolic function in the hippocampus, in close correlation with compromised cognitive function, suggesting a role for oxidative stress. This data supports an impairment of neurovascular response in connection with cognition decline due to oxidative environment-dependent compromised nitric oxide (NO) signaling from neurons to vessels during aging.

Oxidative stress and the potential role of reactive oxygen species in the initiation and progression of systemic sclerosis vasculopathy was reviewed by Abdulle et al. These authors suggest the use of oxidative stress related read-outs as clinical biomarkers of disease activity and evaluate potential anti-oxidative strategies in systemic sclerosis (Abdulle et al).

The synergistic contributions of redox-inflammatory processes for endothelial dysfunction in diabetic retinopathy were discussed by Santiago et al. with emphasis in the endothelial cell communication with other retinal cells.

The role of reactive oxygen species in the development of pulmonary vasculature changes under hypoxic conditions was discussed by Siques et al.

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In different chapters, authors suggest and discuss some therapeutic strategies to protect the vasculature against oxidative stress. Therapeutic approaches such as policosanol consumption and melatonin have been investigated and discussed. The physiological policosanol consumption resulted in significant lowering of brachial and central aortic blood pressure in a dose-dependent manner accompanied by an improvement in lipid profile resulting in improved anti-oxidant and anti-glycation activities in healthy Korean subjects with pre-hypertension (Kim et al). Melatonin and its therapeutic potential was discussed by Guo et al. on cerebral autoregulation following subarachnoid hemorrhage.

Environmental stress factors are important both in adults and in early life. Exposure to stressful environments produced persistent increases in plasma corticosterone and reductions of brain and cardiac NO production followed by a delayed decrease in the NO-dependent component of endothelium-dependent relaxation—changes that collectively accelerated blood pressure increases only in young borderline hypertensive rats (Bernatova et al.). In addition, due to the role of macrophages in all stages of the inflammatory response by acquiring distinct functional phenotypes that are directed by the tissue type and environmental cues, Takahashi et al. highlighting the anti-inflammatory effects of AM18W-13a extract to reduce inflammation in murine macrophages.

Importantly, environmental stress factors during fetal and perinatal life can shape the future health of the individual and increase susceptibility to several adult diseases. Suboptimal intrauterine conditions induce alterations in placental redox balance, associated with poor fetal development and low birth weight. Rodríguez-Rodríguez et al. summarize data on specific redox alterations in key cardiovascular control organs induced by exposure to known stress factors in experimental animals and discuss the emerging role of the mitochondria.

This Frontiers Research Topic in oxidative stress is a view into the rich places of research that have yet to be tapped relative to understanding the role oxidative stress plays both in health and disease.

We are grateful to our contributors for sharing their important work.

AUTHOR CONTRIBUTIONS

CS drafted the editorial. RS and GP read and modified the editorial.

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MINI REVIEW
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Vascular Oxidative Stress: Impact and Therapeutic Approaches

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Oxidative stress has been defined as an imbalance between oxidants and antioxidants and more recently as a disruption of redox signaling and control. It is generally accepted that oxidative stress can lead to cell and tissue injury having a fundamental role in vascular dysfunction. Physiologically, reactive oxygen species (ROS) control vascular function by modulating various redox-sensitive signaling pathways. In vascular disorders, oxidative stress instigates endothelial dysfunction and inflammation, affecting several cells in the vascular wall. Vascular ROS are derived from multiple sources herein discussed, which are prime targets for therapeutic development. This review focuses on oxidative stress in vascular physiopathology and highlights different strategies to inhibit ROS production.

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INTRODUCTION

Oxidative stress is an important underlying factor in health and disease. It is originated by an alteration in the balance of reactive oxygen species (ROS) production and antioxidant defense mechanisms (Halliwell, 1994; Betteridge, 2000). Homeostatic ROS concentrations play a crucial role as secondary messengers in many intracellular signaling pathways in both innate and adaptive immune responses (Schieber and Chandel, 2014; Sies, 2017). The problem occurs when ROS bioavailability overtakes the antioxidant defenses. In these conditions, ROS act as destructive agents affecting proteins, lipids and DNA, leading to cellular damage, tissue injury, and inflammation (Halliwell and Gutteridge, 1999). On the other hand, from a mechanistic point of view, oxidative stress may be better defined as a disruption of redox signaling and control. This concept could redirect research to identify crucial perturbations of redox signaling and control and lead to new treatments for oxidative stress-related disease processes (Jones, 2006).

Oxidative stress has been associated with the pathogenesis of several chronic disorders such as neurodegenerative diseases, diabetes, hypercholesterolemia, and atherosclerosis and is a contributory pathogenic factor in obesity-related disorders (Sayre et al., 2001; Schleicher and Friess, 2007; DeMarco et al., 2010; Giacco and Brownlee, 2010; Sena et al., 2013; Bhatti et al., 2017). In addition, vascular oxidative stress is a leading cause in cardiovascular diseases (Li et al., 2014; Förstermann et al., 2017). Elevated oxidative stress leads to vasoconstriction, vascular remodeling, inflammation, and fibrosis (Rodriguez-Porcel et al., 2017). Insufficient cellular protection against oxidative stress has been described as a major factor for the development of vascular diseases (Li et al., 2014). Oxidative stress is also the principal cause of epigenetic changes that occur during aging (Guillaumet-Adkins et al., 2017).

The involvement of reactive oxygen and nitrogen species has been extensively studied in various pathological conditions. Its etiology is diverse.

This review focuses on the oxidative processes that occur mainly in the vasculature and lead to vascular dysfunction.

Vascular Oxidative Stress

Regulation of vascular tone is critical for the homeostatic function of blood vessels and blood supply to peripheral organs. In physiological conditions, maintenance of appropriate endothelial function provides vasorelaxant properties through release of vasoactive substances (Sena et al., 2013). An imbalance between production of vasoprotective and vasorelaxant factors and vasoconstrictor substances by the endothelium is a hallmark of endothelial dysfunction, which precedes numerous vascular pathologies (Sena et al., 2013). In this context, oxidative stress has a crucial role in the initiation and progression of endothelial dysfunction and vascular disease affecting several cells in the vascular wall (Giacco and Brownlee, 2010; Sena et al., 2013; Rodriguez-Porcel et al., 2017).

In addition, vascular oxidative stress promotes systemic inflammation via immune activation. Activated immune cells migrate into the vasculature, and release several factors including ROS, metalloproteinases, cytokines, and chemokines that promote dysfunction and cause vascular damage promoting vasoconstriction and remodeling of blood vessels (Zhou et al., 2017; Norlander et al., 2018). Vascular remodeling, stiffness, structural elastin abnormalities, and increased oxidative stress are hallmarks of vascular damage in hypertension (Martínez-Revelles et al., 2017).

Endothelium

Endothelial cells regulate vascular tone having a crucial role in the control of organ vascular resistance. These cells, through their secretome, influence vascular smooth muscle cells (VSMCs) and circulating cells such as platelets and monocytes (Sena et al., 2013; Rodriguez-Porcel et al., 2017).

The arterial endothelium is subjected to various injurious stimuli such as oscillatory shear stress, disturbed turbulent flow and oxidative stress among others. The major ROS produced in response to several stimuli (such as hyperglycemia, hyperlipidemia, and hypertension) is superoxide anion ($O_2^{\bullet -}$) that quickly combines with NO to produce peroxynitrite decreasing NO bioavailability and leading to endothelial dysfunction (Sena et al., 2008, 2011). In response, endothelial cells became activated, produce vasoconstrictor agents (thromboxane A2, endothelin-1, or prostaglandin H2) and an inflammatory response is initiated. The endothelium starts expressing adhesion molecules and secretes chemokines such as chemokine (C-C motif) ligand 2 (CCL2) to attract immune cells (Sena et al., 2013). The increased expression of chemokines and proteases in endothelial cells creates a vicious circle perpetuating the inflammatory response (Campbell et al., 2015). During prolonged vascular inflammation, several changes such as an increment in apoptotic cells, remodeling of the extracellular matrix, breakdown of elastic lamella, and endothelial dysfunction, emerge in atheroprone vessels and accelerate atherosclerosis-related complications (Weber and Noels, 2011). Dysfunctional endothelium is crucial to initiate vascular dysfunction leading to several pathologic conditions including macro (atherosclerosis) and microvascular diseases.

Vascular Smooth Muscle Cells

Vasoactive substances exert their vasorelaxant or vasoconstrictive properties through effects on receptors of VSMCs, which are central for the regulation of vascular tone. These substances derive from endothelial cells, vasoactive nerves, and perivascular tissue (Giacco and Brownlee, 2010; Majesky, 2015).

Vascular smooth muscle cells can also be sources of ROS promoting oxidative stress (Lim and Park, 2014). Various stimuli including increased cyclic stretch can promote oxidative stress in VSMCs. It was recently described that lysyl oxidase (LOX), an elastin crosslinking enzyme, is as a novel source of vascular ROS. LOX-derived ROS activate p38 mitogen-activated protein kinase critically influencing elastin structure and vessel stiffness in hypertension (Martínez-Revelles et al., 2017).

Activation of oxidative stress and inflammation occurs via receptors leading to changes in the balance between vasodilators and vasoconstrictors affecting vascular tone and ultimately lead to vascular dysfunction (Bhatt et al., 2014; Lim and Park, 2014). Vascular ROS effects are mediated through redox-sensitive signaling pathways. ROS regulate protein kinases, phosphatases, mitogen-activated protein kinases, and transcription factors; playing an important role as modulators of $[Ca^{2+}]_i$, rho-associated coiled-coil protein kinase (ROCK), and the contractile machinery. Oxidative stress will activate protein kinase C (PKC) leading to the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and in turn increment oxidative stress. PKC activation will phosphorylate and activate multiple mechanisms that ultimately promote VSMCs contraction. Increased Nox-derived ROS generation enhances calcium signaling, up-regulates ROCK and modulates the actin cytoskeleton, thereby promoting vascular contraction and increasing vascular tone. PKC and ROCK activity play a role in exacerbated vasoconstriction associated with vascular dysfunction (Liu and Khalil, 2018).

Adventitia and Perivascular Adipose Tissue

The adventitia surrounds the tunica media containing many different cell types in close continuity with perivascular tissue (Majesky, 2015), particularly in large arteries, influencing both endothelial (through vasa vasorum) and VSMCs. In this context, perivascular adipose tissue (PVAT) is an important regulator of vasculature, with much more than a supportive and mechanical role it has also endocrine and paracrine functions. Visceral adipose tissue is a known source of adipokines but PVAT is also an active producer of both adipokines and inflammatory cytokines (Omar et al., 2014).

Under physiological conditions, PVAT has anti-contractile and anti-inflammatory properties that protect blood vessels. Recent studies have suggested that aortic PVAT is protective for endothelial dysfunction in hypercholesterolemic LDL

receptor knockout mice. Elevated eNOS-derived NO production in aortic PVAT of this animal model is an adaptive mechanism that may protect endothelial function and maintain normal endothelium-dependent relaxation in the early stages of atherosclerotic disease (Baltieri et al., 2018).

In pathologic conditions such as obesity and atherosclerosis, PVAT changes its phenotype and can contribute to vascular oxidative stress because they have an increment in NADPH oxidases in adipocytes with increase activity in obesity-related conditions (Gao et al., 2006; Gil-Ortega et al., 2014; Padilla et al., 2015). In fact, an increment in ROS has been previously described (DeMarco et al., 2010; Salgado-Somoza et al., 2010; Meijer et al., 2011; Sacks and Fain, 2011). In addition, oxidative stress in adipocytes subsequently stimulates recruitment of immune cells (Chatterjee et al., 2009). Perivascular adipocytes also express angiotensinogen (Serazin et al., 2004) and PVAT produces and releases angiotensin II and related peptides (Bujak-Gizycka et al., 2007).

In obesity, PVAT displays hypoxia, inflammation, and oxidative stress culminating in a dysregulated production/secretion of adipokines and cytokines and leading to PVAT changes toward a proinflammatory phenotype. In animal models and in patients with obesity, PVAT phenotype has been described by up-regulation of pro-inflammatory adipokines/cytokines (e.g., leptin, interleukin-6, tumor necrosis factor-α) and down-regulation of anti-inflammatory adipokines/cytokines (e.g., adiponectin and Interleukin-10) (Henrichot et al., 2005; Chatterjee et al., 2009; Marchesi et al., 2009; Ketonen et al., 2010; Meijer et al., 2011; Aghamohammadzadeh et al., 2016; Xia et al., 2016; Sena et al., 2017). In addition, an increment in esterified fatty acids has also been observed (Ghorbani et al., 1997).

The oxidative stress and inflammation in PVAT have a major impact on endothelial function (Wimalasundera et al., 2003; Payne et al., 2010; Sena et al., 2017), vascular stiffness (Tsioufis et al., 2007; Wu et al., 2016), smooth muscle migration (Barandier et al., 2005) and ultimately lead to vascular disease (Marchesi et al., 2009; Ketonen et al., 2010; Ma et al., 2010; Almabrouk et al., 2014; Gil-Ortega et al., 2014; Omar et al., 2014; Molica et al., 2015) reinforcing the "vasocrine" effects of PVAT in obesity-related diseases.

Sources of Reactive Oxygen Species

In biological systems most ROS are generated from mitochondria (Cadenas and Davies, 2000). Mitochondrial dysfunction is an important cause in the development and progression of several diseases: energy surplus and oxidative stress causes the mitochondrial dysfunction fostering ROS production and oxidative stress. Other sources include NADPH oxidase, xanthine oxidase, cytochrome P450, uncoupled endothelial nitric oxide synthase (eNOS enzyme produces $O_2^{\bullet-}$ instead of NO), myeloperoxidases and lipoxygenases (Madamanchi et al., 2005; Santilli et al., 2015) (**Figure 1**).

PRO-OXIDANT PATHWAYS

NADPH Oxidase

NADPH oxidases (Nox) are a crucial contributor to oxidative stress in vascular cells, including endothelial cells, VSMCs, fibroblasts, and perivascular adipocytes (Griendling et al., 2000; Cai et al., 2003). Nox expression and activity are closely linked with clinical risk factors for atherosclerosis (Guzik et al., 2000). There are at least seven variations of the Nox, characterized by their different catalytic subunits (Guzik and Touyz, 2017).

In blood vessels, Nox1 expression is residual under basal conditions and increases considerably after stimuli (Lassègue and Clempus, 2003). Nox2 generates both $O_2^{\bullet-}$ and H_2O_2 directly affecting both NO bioavailability and contractile properties of the vasculature (Judkins et al., 2010; Guzik and Touyz, 2017). Nox4 is expressed in all vascular and some perivascular cell types. Nox4 possesses vasorelaxant properties via eNOS activation. It predominantly produces H_2O_2 and only small amounts of $O_2^{\bullet-}$. In the context of atherosclerosis, Nox4 expression appears to exert vasoprotective effects (Ellmark et al., 2005; Guzik and Touyz, 2017). Nox4 was shown to exert both a beneficial as well as detrimental effect (Lener et al., 2009; Kozieł et al., 2013), depending on the cell context and stimuli that influence its activity. Indeed, Nox4 is a source of ROS, which changes the redox-state of numerous proteins, and further research is needed to clarify its role in the vasculature. Nox 5 is also expressed in blood vessels. It is a Nox sensitive calcium isoform that produces $O_2^{\bullet-}$ (Montezano et al., 2018). Nox 5 is a pro-contractile Nox isoform important in redox-sensitive contraction. It was recently described a novel function for vascular Nox5, linking calcium and ROS to the pro-contractile molecular machinery in VSMCs (Montezano et al., 2018). Further studies are necessary to clarify Nox5 functions.

The renin-angiotensin system also stimulates NADPH oxidase activity contributing to oxidative stress, endothelial dysfunction, and structural vascular changes typical of hypertension and atherosclerosis (Dzau, 1987; Heart Outcomes Prevention Evaluation Study Investigators et al., 2000; Guzik and Touyz, 2017).

The Mitochondrial Respiratory Chain

Mitochondrial oxidative phosphorylation produces $O_2^{\bullet-}$, which is transformed to H_2O_2 by the manganese-dependent superoxide dismutase and subsequently to water by glutathione peroxidase 1 (Boveris et al., 1976). Under pathological conditions, due to insufficient ROS detoxification or excessive ROS production mitochondrial oxidative stress arises (Madamanchi et al., 2005; Yu et al., 2012; Murphy et al., 2016). Several diseases including atherosclerosis in human have been linked with mitochondrial dysfunction and subsequent oxidative stress (Corral-Debrinski et al., 1992).

Xanthine Oxidase

Endothelium dysfunction is linked with an increment in endothelial xanthine oxidase expression (Landmesser et al., 2002;

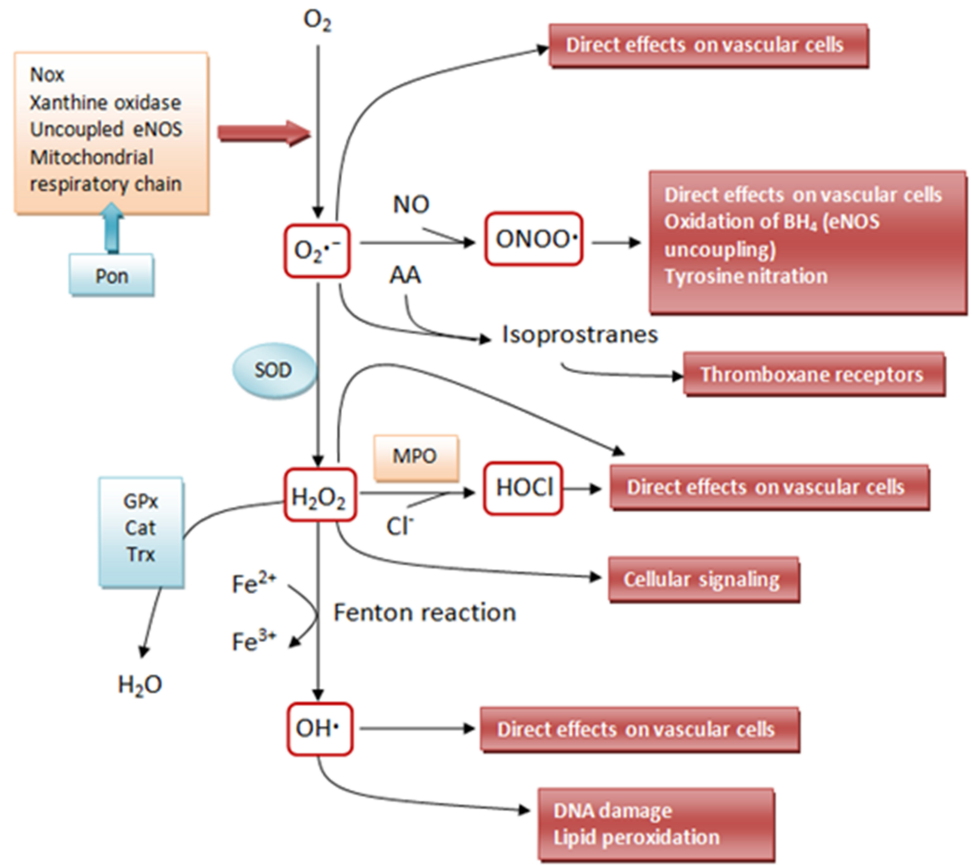

FIGURE 1 | Schematic outline of the interrelationships between some of the more relevant reactive oxygen species (ROS) that affect the vascular wall. Superoxide ($O_2^{-\bullet}$) is produced from molecular oxygen (O_2) by different sources such as nicotinamide adenine dinucleotide phosphate – NADPH oxidase (Nox), mitochondrial respiratory chain, xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS) and lipoxygenases. Superoxide can directly affect vascular cells but can also be converted by superoxide dismutases (SOD) to hydrogen peroxide (H_2O_2). H_2O_2 can undergo spontaneous conversion to hydroxyl radical (OH•, extremely reactive – attacks most cellular components) in the presence of iron (Fe^{2+}) via the Fenton reaction. H_2O_2 produces direct effects on the vascular wall or can be detoxified via glutathione peroxidase (GPx), catalase (Cat), or thioredoxin (Trx) peroxidase to H_2O and O_2. Superoxide can also react with nitric oxide (NO) or arachidonic acid to form peroxynitrite ($ONOO^{-\cdot}$) or isoprostanes, respectively. In addition to other signaling effects, H_2O_2 can activate Nox, resulting in further production of superoxide. The enzyme myeloperoxidase (MPO) can use H_2O_2 to oxidize chloride to the strong-oxidizing agent hypochlorous acid (HOCl). HOCl can chlorinate and thereby inactivate various biomolecules including lipoproteins and the eNOS substrate L-arginine. Besides HOCl generation, myeloperoxidase can oxidize (and thus inactivate) NO to nitrite (NO_2^-) in the vasculature. Paraoxonase (PON) isoforms 2 and 3 can prevent mitochondrial O_2•– generation (Adapted with modifications from De Silva and Faraci, 2017).

Spiekermann et al., 2003) due to the increased production of superoxide and H_2O_2. The activity of this enzyme is increased in patients with coronary artery disease (Landmesser et al., 2007) and inhibitors of this enzyme decrement endothelial dysfunction in both humans and animal models (Guthikonda et al., 2003; Schröder et al., 2006; Nomura et al., 2014).

Uncoupled eNOS

Uncoupled eNOS generates $O_2^{\bullet -}$ instead of NO due to low levels of its cofactor tetrahydrobiopterin (BH_4) or its substrate L-arginine (Alp and Channon, 2004). ROS, in particular peroxynitrite, promote eNOS "uncoupling" (Sena et al., 2013; Santilli et al., 2015). Superoxide reacts with NO forming peroxynitrite that further oxidizes BH_4 to dihydrobiopterin (BH_2), creating a vicious circle and more eNOS uncoupling (Li and Forstermann, 2014). Under physiological conditions, PVAT prevents eNOS uncoupling (Ebrahimian et al., 2009).

Myeloperoxidase

Myeloperoxidase (MPO) is an enzyme that belongs to the mammalian heme peroxidase superfamily, present in polymorphonuclear neutrophils and in monocytes/macrophages (Lefkowitz et al., 2010). MPO produces various compounds with pro−oxidant properties contributing to oxidative stress by oxidizing LDL and lowering NO bioavailability (Pitanga et al., 2014). This enzyme is involved in the formation of products derived from the oxidation of arachidonic acid that are involved in the inflammatory response and in lipid peroxidation (Zhang et al., 2002; Kubala et al., 2010). In addition, MPO promotes atherogenesis through the production of modified subtypes of LDL and HDL lipoproteins

(Daugherty et al., 1994; Nicholls and Hazen, 2009; Kettle et al., 2014).

Lipoxygenases

Lipoxygenases (LOXs) are intracellular enzymes that peroxidize polyunsaturated fatty acids into bioactive lipids with a potential important role in the pathogenesis of atherosclerosis. LOXs, in particular 5−LOX and 12/15 LOX were found to be overexpressed in advanced atherosclerotic lesions. 5−LOX converts arachidonic acid into leukotriene B4, a potent chemo−attractant and leukocyte activator (Rådmark et al., 2015). However, inconclusive data were obtained with respect to the pathophysiological relevance of this leukotriene signaling in atherosclerosis. Thus, more studies are necessary to clarify this matter (Kuhn et al., 2015).

Antioxidant Defenses

In the vascular wall, the primary antioxidant defense systems to neutralize ROS production are enzymatic detoxifiers such as superoxide dismutases (MnSOD, CuZnSOD, EcSOD), catalase, glutathione peroxidase, paraoxonase, thioredoxin peroxidase, and heme oxygenases (Santilli et al., 2015). In addition, the transcription factor nuclear factor erythroid-2 related factor 2 (Nrf2) has also been shown to play a key role in establishing a cellular anti-oxidant defense mechanism against oxidative stress (Bryan et al., 2013; Lee, 2017) and is consider an important therapeutic target to manage vascular dysfunction (Förstermann, 2008).

THERAPEUTICS

Current pharmacological approaches for prevalent diseases, such as obesity, diabetes, and cardiovascular diseases are limited in efficacy. Many studies with antioxidants have proven unsuccessful in clinical trials (Steinhubl, 2008). Hence, the search for new therapies is very important an emergent in order to improve the health status and increase lifespan of the patients.

Lifestyle Approaches

Lifestyle interventions are capable of reducing body weight through an increment in physical exercise and a reduction in caloric intake. Weight loss by calorie restriction and/or exercise can improve the global health state reducing oxidative stress (Imayama et al., 2012).

Mitochondrial-Targeted Therapies

An important and potentially useful therapeutic approach for diseases associated with an increment in oxidative stress is to target antioxidants (as ubiquinol or α-tocopherol) to the mitochondria (Milagros Rocha and Victor, 2007; Murphy and Smith, 2007) with lipophilic cations such as mitoquinone (MitoQ) or MitoE2 (Murphy and Smith, 2007; Smith et al., 2008). However, some studies revealed that MitoQ may be prooxidant and proapoptotic because its quinone group can participate in redox cycling and superoxide production. In light of these results, studies using mitoquinone as an antioxidant should be interpreted with caution (Doughan and Dikalov, 2007).

In addition, enzymatic systems or cell-permeable cationic peptides (Szeto-Schiller peptides) directed to the mitochondria can also be an alternative therapeutic approach (Szeto, 2006).

The mitochondrial protein $p66^{Shc}$ is fundamental in the homeostasis of mitochondria. Targeting this adaptor is another form of decreasing mitochondrial oxidative stress. $P66^{Shc}$ leads to ROS generation (Camici et al., 2015) and its inactivation may be essential in oxidative stress related diseases (Francia et al., 2004). Previous studies have demonstrated that the expression of $p66^{Shc}$ is reduced by SIRT1 activation, which in turn decrements oxidative stress and endothelial dysfunction (Chen et al., 2013). SIRT mimetics are therefore promising tools to mitigate vascular disease progression.

Nrf2 Activators

Nrf2 is a transcription factor ubiquitously expressed in various tissues (including the vasculature) of human and animal models (Pereira et al., 2017; Suzuki and Yamamoto, 2017). It regulates the expression of several antioxidant and detoxification enzymes by binding to upstream antioxidant response elements (Ungvari et al., 2011; Juurlink, 2012).

This transcriptional factor is crucial in the prevention of oxidative stress related-diseases (Kobayashi and Yamamoto, 2006; Lee, 2017). Nrf2 interacts with antioxidant response element sequences of genes coding for antioxidant enzymes include γ-glutamyl cysteine ligase, NAD(P)H quinone oxidoreductase-1, glutathione *S*-transferase, heme oxygenase-1, uridine diphosphate glucuronosyl transferase, superoxide dismutase, catalase, and glutathione peroxidase-1 having a major role in cellular responses to oxidative stress (Alam et al., 1999).

Nrf2 activators can act through different mechanisms (Niture et al., 2014): they can directly prevent the interacting of Nrf2 with the Nrf2-binding site of Kelch-ECH-associated protein-1 (KEAP1) (as, for instance, ML334) (Jiang et al., 2016), they enhance transcription of Nrf2-targeted antioxidant genes (as berberine) (Imenshahidi and Hosseinzadeh, 2016) or they specifically and reversibly increment Nrf2 half-life (as MG-132) (Dreger et al., 2009). Other compounds promote the release of Nrf2 from KEAP1 through interaction with its cysteine thiol residues (as sulforaphane) (Hong et al., 2005).

Nox Inhibitors and Recouplers of eNOS

Triazolopyrimidines are efficient and selective inhibitors of NADPH oxidase activity. They specifically inhibit NADPH oxidase-derived ROS *in vitro* (ten Freyhaus et al., 2006; Drummond et al., 2011; Santilli et al., 2015) and have great potential in the field of atherosclerosis.

GKT137831, an inhibitor of NOx 1 and Nox4, reduces oxidative stress and diabetic vasculopathy (Gray et al., 2017) and is currently under clinical trial. GLX351322 has been suggested as a therapeutic approach in type 2 diabetes through its selective inhibition of NOX4 (Anvari et al., 2015). In addition, rutin, a glycoside of quercetin, exhibits anti-oxidant and anti-inflammatory properties protecting endothelial dysfunction

through inhibition of NOx4 and the ROS-sensitive NLRP3 inflammasome (Wang et al., 2017).

The aldehyde dehydrogenase 2 and its activator Alda-1 are novel molecules capable of inhibiting toxic aldehydes, decreasing oxidative stress and NADPH oxidase activity. These molecules are able to reverse mitochondria dysfunction having a potential role in atherogenesis (Stachowicz et al., 2014; Yang et al., 2018). Nebivolol inhibits the activity of NADPH oxidase and, in addition, reverses eNOS uncoupling (Munzel and Gori, 2009).

Inhibiting eNOS uncoupling is another strategy capable of reducing ROS. Among the drugs used in the clinical practice, inhibitors of the renin–angiotensin–aldosterone system, statins, metformin and pentaerythritol tetranitrate have been described to prevent or reverse eNOS uncoupling. Other compounds, such as resveratrol, sepiapterin, BH_4, folic acid, α-lipoic acid and AVE3085, improve endothelial function through eNOS recoupling although further studies are needed to validate these compounds (Li and Forstermann, 2014; Xia et al., 2017). Recently, thioredoxin was shown to reverse age-related hypertension and arterial stiffness by improving vascular redox and restoring eNOS function (Hilgers et al., 2017).

Targeting Hyperglycemic Memory

Inhibiting hyperglycemic memory concomitantly with a reduction in oxidative stress is crucial under diabetic conditions. The hyperglycemic memory needs to be reduced in diabetes in order to prevent the progression of diabetic complications (Bianchi et al., 2013). Otherwise, this occurrence will result in a vicious cycle continuously producing ROS and leading to vascular dysfunction. Targeting the incretin pathway with dipeptidyl peptidase inhibitors or glucagon-like peptide receptor-1 agonists was able to reduce advanced glycation end products (AGEs) and ROS downstream events that promote cellular damage (Berezin, 2016). Therapies associated with reversion of hyperglycemic memory deserve further investigation in this field and include soluble RAGE/RAGE antagonists, AGE/methylglyoxal inhibitors, S100 inhibitors, targeting vascular protein lysine acetylation (Carrillo-Sepulveda, 2017; Kraakman et al., 2017), among others.

Anti-diabetic Drugs

Studies both in humans and in animal models revealed that anti-diabetic drugs (such as metformin) are able to decrement oxidative stress and inflammation and ameliorate endothelial function reducing the progression toward atherosclerosis (Sena et al., 2009, 2011; Jenkins et al., 2018). Incretin mimetics, in particular GLP1 agonists and dipeptidyl peptidase-4 (DPP4) inhibitors, are currently anti-diabetic agents with a wide range of beneficial effects that include antioxidant effects. An example is linagliptin, an inhibitor of DPP4 that reduces obesity-related insulin resistance and inflammation by regulating M1/M2 macrophage status (Zhuge et al., 2016). It was recently shown that saxagliptin, an inhibitor of DPP4, prevented coronary vascular stiffness through a mechanism that involved a decrement in AGEs, NF-κB, and nitrotyrosine levels in aortic-banded mini swine (Fleenor et al., 2018).

In obesity, diabetes and hypertension mineralocorticoid receptor antagonism improves endothelial function and seems to be a mediator of the switch from vascular health to disease. In endothelial cells, the mineralocorticoid receptor exerts a protective role on endothelial function. In the presence of cardiovascular risk factors, endothelial mineralocorticoid receptor contributes to endothelial dysfunction through NOX activation, eNOS uncoupling, increased epithelial sodium channel expression, and ICAM1/VCAM1-mediated inflammation. Further studies are necessary to clarify these mechanisms (Bakris et al., 2015; Davel et al., 2017).

PCSK-9 Inhibitors

PCSK9 inhibitors are monoclonal antibodies that bind to and inactivate proprotein convertase subtilisinkexin 9 (PCSK9), a liver enzyme that promotes the lysosomal degradation of LDL receptors in hepatocytes thus increasing the number of LDL receptors in the membrane and LDL-cholesterol uptake. In recent studies, evolocumab for 52 weeks significantly decreased the level of vitamin E in LDL-C and increased vitamin E level in HDL (Blom et al., 2015) revealing antioxidant properties (Sabatine et al., 2017).

Other Pharmacological Approaches

Serotonin in peripheral blood reflects oxidative stress and plays an important role in atherosclerosis highlighting the novel anti-atherothrombotic strategy to mitigate vascular disease (Sugiura et al., 2016). Tropisetron, a 5-HT3 receptor antagonist, can attenuate early diabetes through calcineurin inhibition and by suppressing oxidative stress and some inflammatory cytokines in streptozotocin-induced diabetic rats (Barzegar-Fallah et al., 2015).

More recently, cell-permeable peptides mimicking the kinase inhibitory region of suppressor of cytokine signaling- 1 (SOCS1) regulatory protein emerged as an important antioxidant and anti-inflammatory strategy to limit the progression of diabetic complications (Lopez-Sanz et al., 2018). SOCS-targeted therapies have recently been suggested as potential therapeutic approaches in atherosclerosis and deserve further investigation.

Other approach includes the inhibition of selenoprotein P (SeP). SeP is a liver derived secretory protein that promotes insulin resistance (Misu et al., 2010) and is upregulated in the liver of type 2 diabetic patients. This hepatokine downregulates the metabolic switch, AMP-activated protein kinase (AMPK) (Misu et al., 2010). Recent studies have suggested that SeP regulates cellular metabolism and the development of vascular diseases (Ishikura et al., 2014; Cetindağlı et al., 2017; Mita et al., 2017; Kikuchi et al., 2018). SeP promotes vascular smooth cell proliferation through increased oxidative stress and mitochondrial dysfunction in an autocrine/paracrine manner. Sanguinarine, an orally active small molecule, reduces SeP expression and smooth muscle proliferation, and ameliorates pulmonary arterial hypertension in mice and rats. Thus, it seems that SeP could be a novel and realistic therapeutic target (Kikuchi et al., 2018).

The clinical benefit of these compounds awaits further confirmation. Moreover, novel nanotherapeutic approaches are being developed to target oxidative stress and inflammation (Wang et al., 2018).

Traditional medicinal plants have been used by several civilizations through the years contributing to the notion that natural products are relevant sources of new pharmaceutical compounds. Technological advances are now capable of unravel the value of natural products as novel sources for new drug discovery including their role as antioxidants.

CONCLUSION

Vascular oxidative stress promotes endothelial dysfunction and atherosclerosis progression. In the vasculature, several sources promote an increment in oxidative stress.

Preventing vascular oxidative stress and incrementing NO bioavailability may represent the future therapeutic strategy to mitigate the cardiovascular burden and reduce associated risk factors.

AUTHOR CONTRIBUTIONS

CS wrote the article. AL did bibliographic research. LA did bibliographic research. RS revised the manuscript. GP revised the manuscript.

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REVIEW
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Oxidative Stress: A Major Player in Cerebrovascular Alterations Associated to Neurodegenerative Events

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The brain is one of the most exquisite organs in the body with high metabolic demands, and requires a tight regulation of the surrounding environment. This tight control is exerted by the neurovascular unit (NVU) comprising different cell types, where endothelial cells play the commander-in-chief role. Thus, it is assumable that even slight perturbations in NVU might affect, in some cases irreversibly, brain homeostasis and health. In this line, recent findings support the two-hit vascular hypothesis for neurodegenerative conditions, where vascular dysfunction underlies the development of neurodegenerative diseases, such as Alzheimer's disease (AD). Knowing that endothelial cells are rich in mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, two major reactive oxygen species (ROS) sources, this review aims to gather information on how oxidative stress is in the front line of vascular alterations observed in brain aging and neurodegenerative conditions, particularly AD. Also, a brief discussion about the therapeutic strategies aimed to protect against cerebrovascular diseases is included.

Keywords: neurovascular unit, endothelial cells, oxidative stress, mitochondria, NADPH oxidases, Alzheimer's disease

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INTRODUCTION

The brain integrates and regulates several central and peripheral signals to maintain body homeostasis (Ronnett et al., 2009). So, it is not surprising that the brain is an organ with a high energy demand, although it represents only 2% of the body weight (Mergenthaler et al., 2013). In fact, it is widely known that proper neuronal activity entails high amounts of energy. However, the capability of the brain to store energy is very reduced, requiring a constant supply of energy substrates, namely glucose, through blood flow to fulfill its energy needs (Ohta et al., 1992). For that reason the brain receives about 15% of cardiac output and accounts for 20% of total body oxygen consumption (Moreira et al., 2009; Nunomura et al., 2009; Ronnett et al., 2009). At this point, it is worth mentioning the neurovascular coupling, where neurons, glial cells and blood vessels communicate to each other to regulate cerebral blood flow (CBF) and vessels permeability depending on location and neuronal activity in order to efficiently maintain energy substrates supply to satisfy the metabolic needs (Gordon et al., 2007). Cerebral blood vessels comprise unique properties forming the blood–brain barrier (BBB), a physical barrier that permits

the passage of water, some gases and lipophilic molecules by passive diffusion and the selective transport of certain molecules (e.g., glucose) and protects against external toxins and pathogens. Thus, even slight alterations in BBB properties can be responsible for the onset/progression of neurological diseases. From all the BBB constituents (endothelial, mural and glia cells, astrocytes and macrophages), endothelial cells that form blood vessels, play a major role in BBB proper functioning (Daneman and Prat, 2015). Blood vessels control the influx and efflux transport allowing, for example, the entry of glucose and amino acids from the blood into the central nervous system (CNS) and the removal of specific waste products from the CNS into the blood (Daneman and Prat, 2015). More recently, Kubíková et al. (2017), performed a mapping of brain microvessels obtained from two healthy human adult brain samples and found that in certain brain areas microvessels density is higher than others, which can reflect the different susceptibilities to vascular damage. Even slight alterations in brain vasculature can underlie different neurodegenerative events. Indeed, it is widely described that conditions interfering with brain vasculature, such as stiffening of cerebral arteries or increased vessel tortuosity, caused by diabetes and hypertension among other conditions, can induce BBB breakdown underlying the development of neurodegenerative conditions such as Alzheimer's disease (AD) (Carvalho et al., 2010, 2013, 2014; Steinman et al., 2017). It is also known that in many cases BBB integrity is deeply affected by oxidative stress. In fact, increased reactive oxygen species (ROS) production contribute to endothelium dysfunction and increased permeability of BBB (Enciu et al., 2013). These alterations are mainly attributed to the redistribution and/or altered expression of critical tight junction proteins such as claudin-5 and occludin (Schreibelt et al., 2007; Lochhead et al., 2010).

Although it is widely accepted that vascular changes play a crucial role in neurodegenerative diseases, none of the available therapies are effective when translated to clinical trials revealing some gaps in the mechanisms behind the vascular and tissue brain changes under pathological conditions. Thus, studies aimed to develop new non-invasive techniques to better understand why and when changes occur bring a new hope for the treatment of conditions characterized by cerebrovascular alterations (Vaas et al., 2017). In the next section we will discuss the role of oxidative stress in brain vascular alterations putting the focus on mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox).

MAJOR PLAYERS IN VASCULAR OXIDATIVE STRESS

It is widely known that cells homeostasis depends on the regulated levels of ROS. Low/moderate levels of ROS can act as signaling molecules, which are crucial to maintain normal cells function, while uncontrolled generation of ROS causes oxidative damage contributing to cells dysfunction and damage (Chen et al., 2017).

Physiological ROS levels can play important roles in cerebral vasculature (De Silva and Miller, 2016). Studies performed in animal models show that ROS can contribute to the regulation of brain perfusion through their action in vascular tone control (Miller et al., 2009; Grochowski et al., 2018). Indeed, physiological levels of ROS play a major role as cerebral vasodilators (**Figure 1**). For example, it has been shown that the addition of NADPH, the substrate for Nox, in cerebral vessels *in vitro* and *in vivo* cause hydrogen peroxide (H_2O_2)-dependent vasodilatation (Didion and Faraci, 2002; Park et al., 2004; Miller et al., 2005; De Silva and Miller, 2016).

The neurovascular unit (NVU), containing neurons, astrocytes, pericytes, microglia and endothelial cells, is equipped with a powerful antioxidant defense systems that include glutathione (GSH), glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase (Tayarani et al., 1987; Halliwell, 2001). GSH in particular has been shown to play an important role in the maintenance of BBB integrity (**Figure 1**) (Agarwal and Shukla, 1999). Also, the nuclear factor

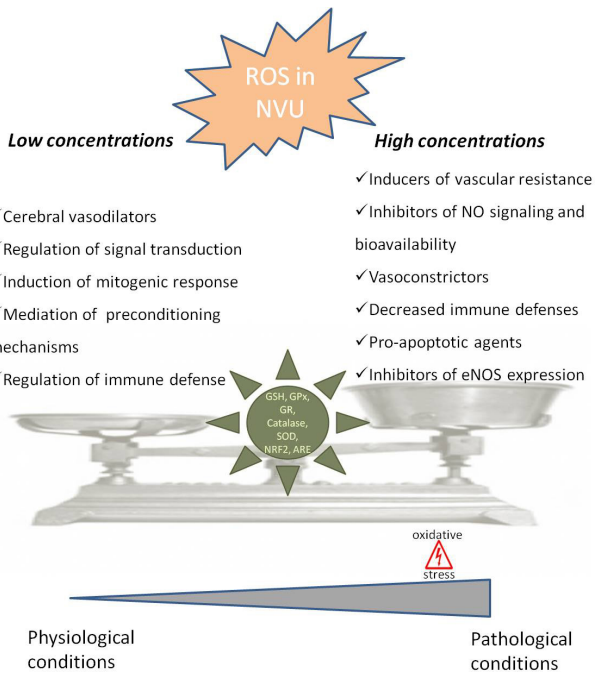

FIGURE 1 | Dual role of reactive oxygen species in neurovascular unit. In neurovascular unit (NVU) reactive oxygen species (ROS) play a controversial role with low/moderate levels of ROS acting as signaling molecules, which are crucial to maintain normal cells function, while uncontrolled generation of ROS causes oxidative damage contributing to cellular dysfunction and cells damage. In physiological situations ROS can act as cerebral vasodilators, regulators of signal transduction, inducers of mitogenic response and immune defense mechanisms, regulators of blood flow and effectors of preconditioning mechanisms. However, when ROS concentrations pass a certain threshold, they lead to an increase in blood flow resistance, decreased nitric oxide (•NO) bioavailability, decreased vasodilatation and immune response, increased apoptosis and decreased endothelial nitric oxide synthase (eNOS) expression, leading to pathological conditions. The delicate balance of ROS levels is regulated by cellular antioxidant systems such as glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase, nuclear factor erythroid 2-related factor 2 (NRF2) and antioxidant response element (ARE).

erythroid 2-related factor 2 (NRF2) seems to play a major defense role by modulating microglial dynamics (Rojo et al., 2010), by protecting astrocytes and neurons from toxic insults (Lee et al., 2003; Vargas and Johnson, 2009) and by regulating the expression of antioxidant enzymes (Shah et al., 2007; Yan et al., 2008). Additionally, NRF2 induces secondary defense proteins via interaction with the antioxidant response element (ARE) in the promoter region of target genes (**Figure 1**). Interestingly, astrocytes have a higher capability of efficiently increase GSH and ARE-linked gene expression, which allows astrocytes to be more protected than neurons against moderate levels of oxidative stress (Vargas and Johnson, 2009).

A failure in the ability of NVU cells to maintain the proper balance between ROS production and their neutralization causes the disruption of NUV and brain homeostasis predisposing to neurodegenerative conditions (Carvalho et al., 2013; Wevers and de Vries, 2016). It has been shown that following an injury microglia and astrocytes produce high levels of ROS via Nox, which seem to have harmful effects in the expression of important molecules involved in BBB integrity (e.g., ZO-1, claudin-5 and occluding). Pericytes, which are in close proximity to endothelial cells, play a vital role in the integrity of BBB (Armulik et al., 2010). Under pathological conditions, pericytes are highly susceptible to oxidative stress (Shah et al., 2013) resulting from overproduction of mitochondrial ROS (Shah et al., 2013). Moreover, it has been demonstrated that ROS production by activated microglia causes pericytes apoptosis (Ding et al., 2017). However, there is a lack of information regarding the role of ROS in pericytes under physiological conditions. Endothelial cells signaling seems to be crucial in the regulation of NVU proper functioning with increased ROS formation playing a major role in the alteration of NVU function and coupling (Girouard and Iadecola, 2006; Faraco et al., 2016).

It was also shown that ROS-induced changes in microcirculation can have profound implications in brain vascular pathophysiology due to alterations in blood flow resistance and, consequently, in regulation of blood pressure (**Figure 1**) (Staiculescu et al., 2014). Both superoxide ($O_2^{\bullet -}$) and H_2O_2 are able to cause both relaxation as well as contraction of cerebral blood vessels depending on the concentration and presence of other species (Faraci and Sobey, 1998; Allen and Bayraktutan, 2009; Freeman and Keller, 2012). In fact, oxidative stress can impair cerebral vascular function via the disruption of endothelium-dependent nitric oxide ($^{\bullet}$NO) signaling (**Figure 1**) (Girouard et al., 2007; Mayhan et al., 2008; Miller et al., 2010b). It is widely described that the reaction of $O_2^{\bullet -}$ with $^{\bullet}$NO leads to a decrease in $^{\bullet}$NO bioavailability and, consequently, a decrease in its vasodilator, anti-proliferative, and anti-inflammatory properties (De Silva and Miller, 2016). Furthermore, increased levels of H_2O_2, a common vasodilator in cerebral circulation, can act as a pro-apoptotic agent in cerebral vascular cells (**Figure 1**) (Li et al., 2003). Increased levels of ROS can also lead to an increase in Rho kinase signaling (Aghajanian et al., 2009) interfering with endothelial nitric oxide synthase (eNOS) expression and activity, affecting $^{\bullet}$NO production (**Figure 1**) (Faraco et al., 2013).

Considering vascular cells, there are several ROS sources such as mitochondrial electron-transport chain, cyclooxygenases (COXs), lipoxygenases, cytochrome P450 reductases, xanthine oxidase, nitric oxide synthase (NOS) and Nox (Miller et al., 2010a). However, in this review, we only discuss mitochondria and Nox, both of them widely described as main producers of ROS either in physiological and pathological conditions in endothelial cells.

Mitochondria: More Than an Energy Producer

Reporting to the history of biology evolution, mitochondria are described as organelles derived from aerobic bacteria that in ancient times invaded proto-eukaryotic cells as parasites (Dromparis and Michelakis, 2012). From there, a symbiotic relationship evolved with mutual benefits and mitochondria became intracellular organelles (Dromparis and Michelakis, 2012). Although mitochondria are widely described as energy producers through the highly conserved oxidative phosphorylation (OXPHOS) process (Cadenas and Davies, 2000; Chan, 2006), nowadays the role of mitochondria reached substantially higher importance in cell homeostasis due to their involvement in several vital processes such as cell growth and differentiation, cell cycle control and death (Osellame et al., 2012; Carvalho et al., 2015), intermediary metabolism, calcium (Ca^{2+}) homeostasis and signaling, and apoptosis (Chan, 2006).

However, mitochondrial energy production can be a double-edge sword. Indeed, OXPHOS is not 100% efficient and during this process an electron leak occurs between mitochondrial complexes leading to the production of ROS such as $O_2^{\bullet -}$, $^{\bullet}$NO, hydroxyl radical ($HO^{\bullet}$), peroxynitrite ($ONOO^-$), and H_2O_2 (Richter and Kass, 1991; Ricquier and Bouillaud, 2000; Goetz and Luch, 2008). As previously mentioned, low/moderate ROS levels exert a beneficial role by activating protective mechanisms (Valko et al., 2007; Correia et al., 2010; Alfadda and Sallam, 2012). However, when ROS levels reach critical values they lead to oxidative stress and activate anomalous signaling mechanisms that can lead to cells degeneration and death (Brown and Borutaite, 2001; Sheu et al., 2006).

Brain endothelial cells seem to possess a number of mitochondria higher than that observed in peripheral endothelial cells (Oldendorf et al., 1976; Alyautdin et al., 2014). However, in comparison with other cell types with higher energy requirements, mitochondria content in endothelial cells is modest. In rodent models, mitochondria compose 2–6% of the cell volume as opposed to 28% in hepatocytes and 32% in cardiac myocytes (Dromparis and Michelakis, 2012; Kluge et al., 2013). These observations support the idea that mitochondria is not a major source of energy in brain endothelial cells. In fact, several studies support the idea that brain endothelial cells obtain a large proportion of their energy from anaerobic glycolytic metabolism of glucose (Spahr et al., 1989; Mertens et al., 1990; Culic et al., 1997). Actually, mitochondria are more likely to serve primarily as essential signaling organelles in the vascular endothelium (Quintero et al., 2006; Tang et al., 2014; Busija et al., 2016).

The activation of mitochondria by physiological stimuli or pharmacological agents leads to the liberation of vasoactive factors by the endothelium, which exert a major role in the modulation and maintenance of BBB integrity and brain homeostasis (Busija and Katakam, 2014). Moreover, it is far known that aging leads to a decrease in mitochondria number in cerebral endothelial cells associated with the loss of BBB integrity (Mooradian, 1988). The BBB high demand of mitochondrial activation comes with a price, the prospect for an increased ROS production that in physiological conditions is regulated by antioxidant enzymes such as glutathione reductase, manganese superoxide dismutase, catalase, among others (Freeman and Keller, 2012). Additionally, it has been demonstrated that vascular endothelial growth factor (VEGF) exerts its functions in endothelial cells migration through mitochondrial ROS (Wang et al., 2011), a process involved in several physiological processes such as wound healing and vascular repair (Freeman and Keller, 2012). Moreover, studies in human coronary resistance arteries showed that mitochondrial ROS are involved in endothelium regulation of vascular homeostasis (Liu et al., 2003). In the same study, the authors reported that mitochondrial ROS have an influence in vascular regulation and health. Indeed, the authors tested mitochondrial complexes I and III and Nox inhibitors and observed that only the mitochondrial inhibitors were able to exert effects in the regulation of flow-induced dilation (Liu et al., 2003). Besides the above evidence more studies are needed to explore the role of mitochondrial ROS in cerebral vasculature during disease progression, in contrast to the extensive literature concerning systemic vessels.

NADPH Oxidases

The Nox family is also broadly studied due to its main catalytic function of ROS production by transference of an electron to molecular oxygen (Takac et al., 2012). The Nox family is composed by different isoforms: Nox1 to 5, Duox1 and Duox2 with a broad expression in different organs and tissues and with different cellular locations at vascular walls (de Almeida et al., 2017). Except for Nox-5 and DUOX-1 and -2, Nox are phagocytic oxidases, whose main task is to generate ROS to kill foreign pathogens at homeostasis. Of note, the major source of endothelial cells ROS comes from NOX-1, -2, -4, and -5 (Pendyala et al., 2009; Drummond and Sobey, 2014). Different Nox isoforms seem to produce different forms of ROS; Nox1 and 2 mainly produce $O_2^{\bullet -}$; Nox4 mainly produces H_2O_2 and Nox5 seems to produce both $O_2^{\bullet -}$ and H_2O_2 (Dikalov et al., 2008; Helmcke et al., 2009). Under physiological conditions, Nox-derived ROS seem to exert an important role in the regulation of vasodilatation (Togliatto et al., 2017). Although it is considered that under physiological conditions Nox activity is constitutively low, when its function increases with the consequent increase in ROS production, it can trigger ROS production by other sources (Landmesser et al., 2003). Indeed, it has been described that Nox-derived ROS play a major role in coordinating some physiological processes such as innate immunity, modulation of redox-dependent signaling cascades, and can act as cofactors in the production of hormones (Drummond et al., 2011). Furthermore, it is described that H_2O_2 produced by Nox family can exert an endothelium-derived hyperpolarizing role causing vasodilatation and reducing blood pressure in mice (Ray et al., 2011). The Nox activity depends on the stimuli, such as cytokines (De Keulenaer et al., 1998), growth factors (Brandes et al., 2001), hyperlipidemia, and high glucose (Jansen et al., 2013). Additionally, Nox family is known for its role as oxygen sensors, modulating the different responses to hypoxia through hypoxia-inducible factor 1 alpha (HIF-1α) mRNA induction and HIF-1α stabilization (Gorlach et al., 2001), a process that is responsible for alterations in gene expression due to the oxidation of target proteins.

However, the overproduction of ROS by Nox family plays a major role in disruption of vascular homeostasis, which can underlie the development of neurodegenerative diseases. Of notice, so far only Nox2 and Nox4 were associated with endothelium dysfunction probably due to their major role as vascular ROS producers, in comparison to the other Nox isoforms (Matsuno et al., 2005; Takac et al., 2012).

Compelling evidence shows that Nox-derived oxidative stress causes many of the deleterious effects of angiotensin II on the cerebral vasculature. It was observed that angiotensin II acutely and chronically, increases $O_2^{\bullet -}$ production by Nox in rodent cerebral vessels (Girouard et al., 2006, 2007; De Silva and Faraci, 2016). Also, functional alterations on rodents' cerebral arterioles following angiotensin II treatment are prevented by co-treatment with the ROS scavenger MnTBAP and the Nox2 peptide inhibitor gp91ds-tat (Girouard et al., 2006). It was also reported that angiotensin II can also activate and increase $O_2^{\bullet -}$ production in cerebral vessels by activating Nox1 (Jackman et al., 2009).

Interestingly, although not extensively studied, the Nox family activation and expression seems to be seasonal, a fact that could be correlated with seasonal alterations in oxidative stress and endothelium dysfunction observed in humans and rats unveiling a new strategic pathway to future studies of clinical relevance (Hopkins et al., 2011; Konior et al., 2014).

AGING AND NEURODEGENERATIVE DISORDERS: WHEN THINGS START TO FAIL

The aging process causes several alterations in brain blood vessels such as decreased elasticity, increased cerebrovascular remodeling and calcification, gradual cerebrovascular wall stiffness, low-grade and widespread inflammation and oxidative stress (Camici et al., 2015), factors that increase the risk for cerebrovascular diseases. So far, there is no agreement of how aging process occurs although several theories can be found in the literature. One of the most accepted theory for aging was first proposed by Harman (1956) and is known as "free radical theory of aging," which postulates that ROS levels increase with age, being responsible for deoxyribonucleic acid (DNA), proteins and lipids oxidative damage (Harman, 1956).

Recent findings demonstrated the existence of BBB permeability alterations during normal aging in human hippocampus using advanced dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI)

(Montagne et al., 2015). It is also known that aging increases BBB susceptibility to different challenges and its permeability is less controlled allowing the entrance of neurotoxic substances, which affect brain homeostasis (Zlokovic, 2011; Caplan et al., 2017). In fact, BBB dysfunction is more pronounced under pathological conditions, including in mild cognitive impairment (MCI) individuals, supporting the idea that vascular alterations are early events in cognitive deficits (**Figure 2**) (Montagne et al., 2015). Using a multiphoton microscope, Han et al. (2015) observed increased levels of $O_2^{\bullet -}$ in the cerebral vessels of aged PS1/APP transgenic mice, a model of AD. Also, electron and confocal microscopy studies revealed that in F344 rats aortas, the expression of the mitochondrial biogenesis factors, such as mitochondrial transcription factor A and peroxisome proliferator-activated receptor-γ coactivator-1-alpha (PGC-1α), decreases with aging (Ungvari et al., 2008). Moreover, an increase in mitochondrial H_2O_2 production was observed in aged arterial rat vessels leading to an activation of nuclear factor-κB (NF-κB) and induction of inflammatory phenotypic changes in aged vasculature (Ungvari et al., 2007). Furthermore, an age-dependent decline in mitochondrial complexes I, III, and IV subunits expression was observed in rat aortas, although the authors did not specify if all the subunits of the three complexes were evaluated or not (Ungvari et al., 2008). Also, Wenzel et al. (2008) were able to show an age-dependent increase in mitochondrial ROS production and mitochondrial DNA lesions causing aorta vascular dysfunction in two different mice models, the ALDH-2–/– and MnSOD–/– models, which are deficient in aldehyde dehydrogenase-2 (ALDH-2) and manganese superoxide dismutase (MnSOD), respectively. Furthermore, the *in vitro* use of mitochondrial complex I inhibitor rotenone, after angiotensin II-induced $O_2^{\bullet -}$ production, showed significant improvements in endothelial dysfunction and tolerance in human aortic endothelial cells (Dikalov et al., 2014; Mikhed et al., 2015). Furthermore, the use of preconditioning strategies as a therapeutic intervention is emerging and under extensive scrutiny. Indeed, studies from our laboratory were able to show that treating rat brain endothelial cells with non-lethal cyanide concentrations, which lead to increased mitochondrial ROS production, was able to confer protection against *a posterior* high glucose-mediated damage, preventing apoptotic cell death (Correia et al., 2012). Importantly, Correia et al. (2012) were able to prove that in the absence of mitochondrial DNA, using mitochondrial-depleted DNA human teratocarcinoma NT2 cells, this effect was abrogated, emphasizing mitochondrial role in endothelium protection by preconditioning (Correia et al., 2012). Moreover, the use of mitochondrial-targeted antioxidants such as MitoTempo, showed promising results in counteracting age-related and microgravity-induced and hyperglycemia-induced endothelial dysfunction (Carvalho et al., 2014; Zhang et al., 2014). Indeed, Mitotempo was able to protect endothelial primary cultures of diabetic mouse model (db/db) and rat cerebral arteries against amyloid β (Aβ) toxicity (Carvalho et al., 2014) and lead to improvements in spatial working memory and motor skill learning in young 5xFAD mouse models of AD (Lu et al., 2015). Moreover, it was also able to recover mitochondrial dysfunction observed in rat cerebral arteries of animals exposed to microgravity conditions through reduction of mitochondrial ROS levels, increased mitochondrial potential and improvement in mitochondrial respiratory chain function (Zhang et al., 2014).

Mitochondrial-derived oxidative stress can also activate vascular inflammation in aged carotid arteries and vessels (Csiszar et al., 2007; Ungvari et al., 2007) leading to atherosclerosis through activation of endothelial NF-κB, which is responsible for the upregulation of adhesion molecules and increased monocyte adhesiveness in aged aortic arteries (Ungvari et al., 2007). Moreover, changes in Nox activity and/or expression is widely described in aged brain vessels (Park et al., 2007; Mayhan et al., 2008). Nevertheless, the mechanistic role of Nox in the aging process remains obscure (Sahoo et al., 2016).

Data from the literature also show that in cultured aged cerebromicrovascular endothelial cells (CMVECs) the mRNA expression levels of Nox2 and 4 subunits were upregulated compared with young CMVECs, a phenomenon similar to that observed in the myocardium, leading to increased levels of oxidative stress (Toth et al., 2014). Although further studies must be performed in order to corroborate this hypothesis, it seems that, similarly to the myocardium, in CMVECs the increased expression of Nox 2 and 4 causes oxidative stress, which activates the renin–angiotensin–aldosterone system (RAAS) (Wang et al., 2010). This activation can occur through a ROS-induced increase in angiotensinogen (AGT), a 60-kDa α2-globulin glycoprotein that constitutes the precursor of RAAS; stimulation of renin, the enzyme responsible for the initiation of the RAAS pathway; and release or regulation of angiotensin converting enzyme (ACE) activity, crucial for the formation of angiotensin II, the major effector of RAAS (Morato et al., 2017). Furthermore, since Nox 4 is usually localized in mitochondria, its increased expression can also contribute to increased levels of mitochondrial ROS potentiating the aging process as well as age-related diseases (Sahoo et al., 2016). The use of Nox inhibitors showed promising results in reducing oxidative stress levels and in improving endothelial function in several disease models (Girouard et al., 2006; Kahles et al., 2007; Matsumoto et al., 2007; Miller et al., 2010a). Also the use of resveratrol seems to be effective in restoring cerebromicrovascular endothelial function through a downregulation of Nox-derived ROS production in aged mice (Toth et al., 2014).

Despite the extensive literature stating that age-associated oxidative stress can lead to cerebrovascular dysfunction and cognitive decline, there is a significant gap in our knowledge regarding the exact mechanisms underlying these defects (Tarantini et al., 2017a).

CEREBROVASCULAR OXIDATIVE STRESS AND NEURODEGENERATIVE EVENTS: THE CASE OF ALZHEIMER'S DISEASE

Although the previous alterations seem to be common features in physiological aging of vessels, somewhere over the way those defects can become more pronounced triggering

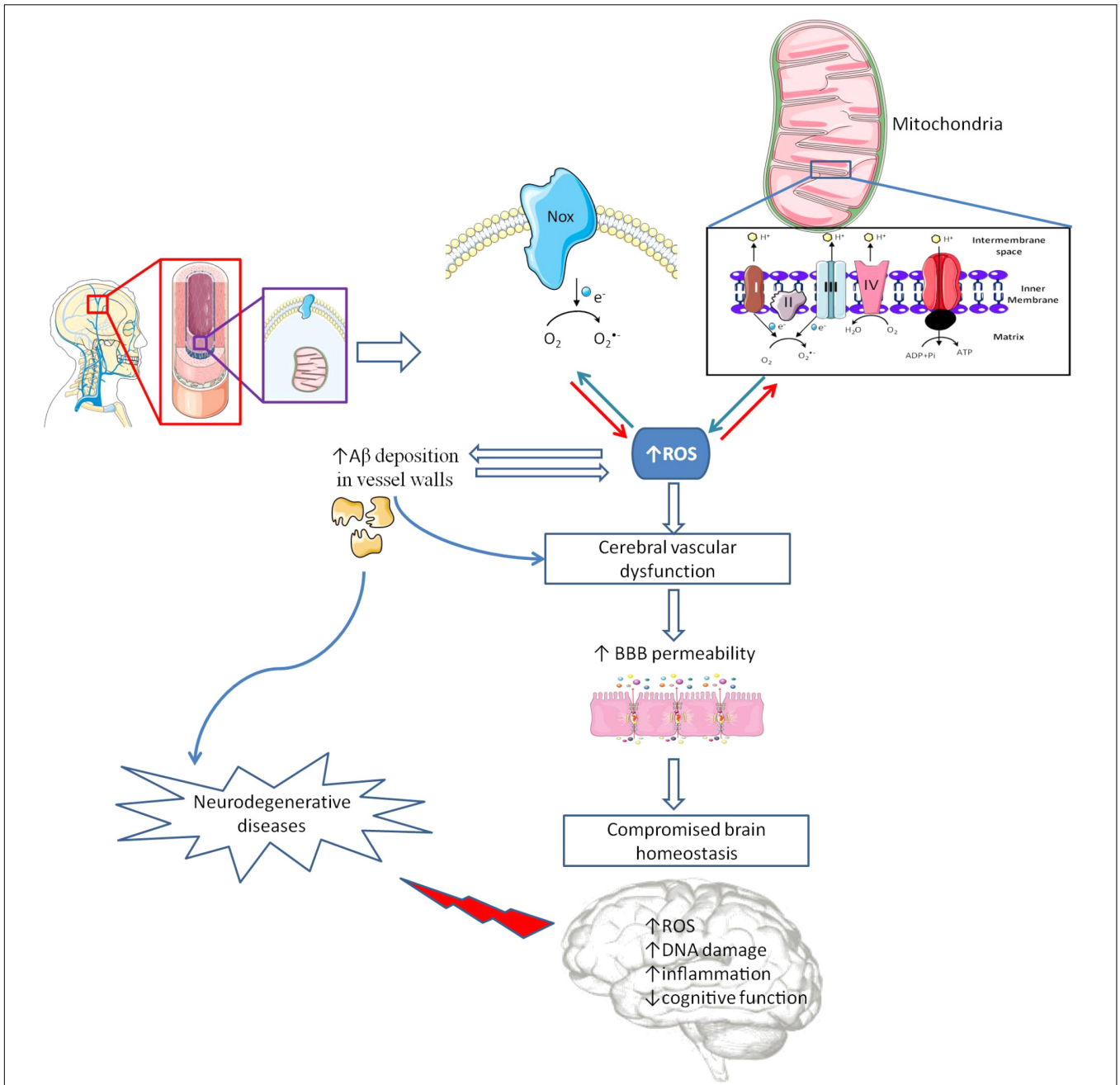

FIGURE 2 | Cerebrovascular alterations and brain damage. The brain is a very delicate organ that relies on an extremely controlled environment and on a constant energy supply due to its high metabolic demand, which depend on blood–brain barrier (BBB) proper functioning. Besides other cell types (astrocytes, pericytes, microglia and a basement membrane made from structural proteins) endothelial cells (EC) assume the major role in controlling BBB integrity. Inside EC, mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) act as main sources of reactive oxygen species (ROS). In pathological conditions, an increased ROS levels cause cerebrovascular dysfunction, which promotes an increase in BBB leakage interfering with brain energy supply and homeostasis, and increasing amyloid β (Aβ) peptide deposition in vascular walls. Alterations in BBB features are associated with oxidative stress and damage and inflammatory processes, among other deleterious alterations, contributing to cognitive defects and, eventually, the development of neurodegenerative diseases.

neurodegenerative events (Kling et al., 2013). Indeed, recent reports suggest a major role for the NVU in the initiation of neurodegeneration (Nelson et al., 2016). In fact, NVU dysfunction leads to an increased BBB permeability with the subsequent entry of neurotoxic molecules into the brain disturbing its homeostasis. A decrease in the removal of neurotoxic substances from the brain, and a deficient nutrient delivery system eventually culminate in neuronal loss and synaptic dysfunction (Zlokovic, 2008). Thus, a decline in cerebrovascular function in age-associated diseases, such as

AD, is easily understandable (Girouard and Iadecola, 2006). Indeed, it is estimated that about 60–90% of AD patients also present cerebrovascular alterations including cerebral amyloid angiopathy (CAA), microinfarcts and ischemic lesions and microvascular degeneration (Jellinger and Mitter-Ferstl, 2003; Bell and Zlokovic, 2009).

It has been recently reported the existence of BBB and vascular alterations in several diseases such as Parkinson's disease (PD), Huntington's disease and cerebral small vessel disease (Lin et al., 2013, 2015; Lee and Pienaar, 2014; St-Amour et al., 2015; Al-Bachari et al., 2017) although there is a gap concerning the mechanisms underlying those alterations and the role of vascular oxidative stress in these pathologies.

Concerning AD, the most common form of dementia in the elderly, more information exists about the vascular alterations that occur in this disease (Gallart-Palau et al., 2016). Several clinical and basic evidence points to the existence of a major contribution of both large artery and small vessel disease in the pathogenesis of AD (Iadecola, 2013; De Strooper and Karran, 2016; Faraci, 2017). Having this into account, the two-hit vascular hypothesis of AD emerged, postulating that cerebrovascular damage is an initial insult that is self-sufficient to initiate neuronal injury and neurodegeneration, but can also promote accumulation of Aβ peptide, a neurotoxic peptide, in the brain (Nelson et al., 2016). Thus, events that compromise vascular health can initiate a cascade of deleterious events that culminate in AD development. In fact, previous studies from our laboratory demonstrated that some risk factors for AD, such as hyperglycemia, increase the susceptibility of brain endothelial cells to Aβ peptide, a phenomenon related to ROS overproduction (Carvalho et al., 2014). Moreover, several genetic [apolipoprotein E4 (APOE4), phosphatidylinositol-binding clathrin assembly protein (PICALM), clusterin, presenilin 1, amyloid precursor protein (APP), mesenchyme homeobox gene 2 (MEOX2)] (Nelson et al., 2016) and non-genetic (hypertension, metabolic syndrome, hypercholesterolemia, atherosclerosis, alcohol and substance abuse, among others) (Kivipelto et al., 2002; Skoog and Gustafson, 2006; Nelson et al., 2016; Campos-Pena et al., 2017) risk factors for AD seem to be linked with alterations in vascular function supporting the idea that vascular alterations play a major role in AD pathology.

Recent studies in wild type mice showed that, even under normal conditions, there is a different regional susceptibility of the cerebrovasculature to oxidative stress (Austin et al., 2015). Vessels from cortex and hippocampus, the two main areas compromised in AD, contain significantly higher levels of intracellular $O_2^{\bullet -}$ and increased protein levels of both Nox 2 and 4 (Austin et al., 2015). Other studies revealed significant changes in blood vessels morphology, decreased vascular density and increased vessels tortuosity in AD brains (Hunter et al., 2012). Moreover, the use of recent technologies such as arterial spin labeling magnetic resonance imaging (MRI), functional blood-oxygen-level-dependent (BOLD)-MRI, fluorodeoxyglucose-positron emission tomography (FDG-PET), and single-photon emission computerized tomography (SPECT), showed decreased levels of CBF in early phases of human AD progression (Daneman and Prat, 2015). Recently, Lourenço et al. (2017) showed that reduced CBF changes found in triple transgenic for AD (3xTg-AD) mice preceded memory dysfunction thus suggesting that cerebrovascular dysfunction could be the primary cause of neurovascular uncoupling in AD. Those observations are supported by MRI, transcranial doppler and SPECT studies revealing different patterns of decreased CBF coincident with the brain regions where neuropathological alterations are more relevant in AD (Hu et al., 2010; Pimentel-Coelho and Rivest, 2012). Those alterations seem to be closely related with the levels of oxidative stress in the cerebrovasculature. Studies from our laboratory showed that 3xTg-AD mice present an increased BBB permeability, in cortex and hippocampus (Carvalho et al., 2013), these alterations being correlated with decreased aconitase activity, an enzyme whose activity is inhibited by $O_2^{\bullet -}$ (Carvalho et al., 2013). The increased levels of ROS appeared to be the result of impaired activity of mitochondrial enzymatic complexes I–III (**Figure 2**). Moreover, and similarly to our observations in mitochondria isolated from whole brain of 3xTg-AD mice (Carvalho et al., 2012), Golgi silver impregnation studies using multiple samples from the hippocampus and cortices of AD patients showed that brain endothelial cells present enlarged mitochondria, disruption of the mitochondrial cristae and reduced abundance (Baloyannis and Baloyannis, 2012).

An interesting fact is that even though mitochondrial morphology is very similar in male and female rodent cerebral arteries, major differences can be observed concerning mitochondrial protein mass, respiration, and function (Rutkai et al., 2015), which is in accordance with sex/gender differences observed in several brain-related studies (Giordano et al., 2013; Christov-Moore et al., 2014; Ingalhalikar et al., 2014; Sun et al., 2015) including the susceptibility to develop AD (Candeias et al., 2017).

Mitochondrial ROS overproduction also increases Aβ deposition in vessels walls, which is a common feature in many cases of AD (**Figure 2**). Indeed, 34–35% of human AD cases present CAA, predominantly in the occipital lobe (Love et al., 2014). Aβ deposition in vessels walls seems to initiate a vicious cycle, Aβ increases ROS levels, which in turn potentiate Aβ deposition (**Figure 2**) (Park et al., 2011). There is also evidence showing that Aβ can induce Nox activation and ROS production, which are responsible for the activation of multiple mechanisms involved in vascular dysfunction and decreased levels of tight junction proteins mRNA (Carrano et al., 2011). However, Nox driven ROS production does not seem to affect Aβ production in Tg2576 mice, a mouse model of AD, lacking the key Nox 2 subunit (Park et al., 2008, 2011).

Endothelial ROS overproduction upregulates the endothelial production and release of endothelin-1, a well know vasoconstrictor that appears to be increased in AD (Palmer et al., 2013). Likewise, ROS overproduction seems to be responsible for reduced $^{\bullet}NO$ bioavailability mainly by its fast reaction with $O_2^{\bullet -}$ generating $ONOO^-$ (Park et al., 2005; Tong et al., 2005) and for a decreased activity of potassium channels (Erdos et al., 2004), two major factors required for endothelial-mediated dilations in brain vasculature (Hamel et al., 2016). Moreover, Aβ-induced nitrosative stress in endothelial cells is also responsible for DNA

TABLE 1 | Antioxidant based therapies.

Molecular target	Compound	Mechanism of action	Reference
Mitochondria	17β-estradiol	↑Mitochondrial biogenesis ↑Antioxidant enzymes	Kemper et al., 2014
	Mitotempo	↑Mitochondrial function ↓Vascular O_2^- ↑Vascular NO production	Pung et al., 2012; Kizhakekuttu et al., 2012; Carvalho et al., 2014; Tang et al., 2014
	MitoQ	↓Oxidative stress ↓Endothelial dysfunction ↓Leukocytes adhesion	Ghosh et al., 2010; Rodriguez-Cuenca et al., 2010; Smith and Murphy, 2011; Gioscia-Ryan et al., 2014
NADPH oxidases	Genetic deletion of Nox 2	Improve cerebral vessels functioning	Park et al., 2007, 2008
	Apocynin Diphenyleneiodonium chloride	↓Nox-derived ROS production ↑eNOS-driven reactivity	Mayhan et al., 2008
	Fulvene–5 triphenylmethane derivatives grindelic acid ML171	Inhibit extracellular Nox domains (under investigation)	Perry et al., 2006; Bhandarkar et al., 2009; Gianni et al., 2010; Munson et al., 2012; Kofler et al., 2013

damage, resulting in poly (ADP-ribose) polymerase (PARP) activation, ultimately leading to a large increase in intracellular Ca^{2+} through transient receptor potential melastatin-2 channels activation (**Figure 2**) (Park et al., 2014).

Reactive oxygen species overproduction also interferes with HIF-1α reducing its expression and activity which will restrain the stimulus to promote angiogenesis and new vessels formation (Ogunshola and Antoniou, 2009) leading to a vicious cycle of impaired capillary perfusion, hypoxia and oxidative stress (Mamelak, 2017).

FINAL REMARKS

Recognizing vascular ROS production as one of the main causes of BBB dysfunction in aging and dementia, particularly in AD, raises the prospect that pharmacotherapy targeting the major ROS producers, namely mitochondria and/or Nox, might open a new venue to stop or, at least, slow dementia progression (Sweeney et al., 2015). Indeed, relying on the overwhelming evidence that mitochondrial dysfunction could be in the genesis of neurovascular dysfunction, several molecules with potential therapeutic effects were designed to target ROS, boost mitochondrial function and decrease free radical production and oxidative damage (Tong et al., 2005; Hamel et al., 2008; Moon et al., 2014) in an attempt to improve neurovascular health. Some of those molecules ameliorated the cognitive performance of mouse models of cerebrovascular disease (Tong et al., 2005). It was observed that the steroid hormone 17β-estradiol (estrogen) modulates the expression of several transcriptional regulators causing a decrease in PGC-1α, and an increase in PGC-1β, PGC-1-related coactivator (PRC), nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM), which protect cerebral blood vessels (**Table 1**) (Kemper et al., 2014). These protective effects were due to increased mitochondrial biogenesis and antioxidant enzymes (Kemper et al., 2014). However, the use of antioxidant therapy in cerebrovascular disease still needs validation. Indeed, several promising approaches failed in clinical trials (Tarantini et al., 2017b), opening an intense debate about the reasons for the failure of those molecules in humans. Merely as an example, the use of tempol showed promising results in improving vasodilator responses of cerebral arterioles in aged APP transgenic mice with CAA (Han et al., 2015) but so far there is no evidence of its efficacy in clinical trials. Furthermore, two major concerns arise from the use of antioxidants as therapeutic approaches: (1) physiological ROS concentrations are important in normal cell functioning and the use of antioxidants may interfere with ROS signaling and (2) the concentrations of antioxidants revealed to be suboptimal doses in certain situations. Indeed, it is known now that supra-physiological concentrations are required to compete with the constant reaction that usually occurs between $O_2^{\bullet -}$ and $^{\bullet}NO$ (Drummond et al., 2011). Large part of the studies failed to prove that antioxidants reach the vasculature at appropriate/therapeutic concentrations (De Silva and Miller, 2016).

To overcome the failure of the traditional antioxidants, researchers are developing new specific-targeted antioxidants. However, evidence about the specific-target antioxidants effects in endothelial cells is scarce. Moreover, mitochondria-targeted antioxidants decrease the dose required and limit toxic side effects rendering them a promising therapeutic approach (Smith and Murphy, 2011). It was observed that MitoTempo, a mitochondria-targeted antioxidant, was able to counteract Aβ-induced damage under hyperglycemic conditions in rat and mouse primary cultures of endothelial cells (Carvalho et al., 2014). Additionally, it was shown that MitoTempo was able to improve mitochondrial function and coronary collateral growth after ischemia/reperfusion in Zucker fatty rats (**Table 1**)

(Pung et al., 2012). Recent studies also showed that MitoTempo was able to decrease vascular $O_2^{\bullet -}$ and increase vascular $^{\bullet}NO$ production improving endothelial-dependent relaxation in angiotensin II-induced hypertensive C57Bl/6 mice (Tang et al., 2014). It was also observed that MitoTempo improves endothelial function and reduces mitochondrial $O_2^{\bullet -}$ levels in subcutaneous arterioles isolated from type 2 diabetic patients (**Table 1**) (Kizhakekuttu et al., 2012). Moreover, MitoQ, another mitochondria-targeted antioxidant was able to reduce oxidative stress, without causing adverse effects, on wild-type mice (Rodriguez-Cuenca et al., 2010; Smith et al., 2011) and animal models of neurodegenerative diseases such as AD and PD (Ghosh et al., 2010; Manczak et al., 2010). MitoQ was able to improve age-related arterial endothelial dysfunction in C57BL/6 mice (**Table 1**) (Gioscia-Ryan et al., 2014). Furthermore, MitoQ has been shown to prevent cocaine-induced cardiac dysfunction in Wistar rats (Vergeade et al., 2010). However, the use of mitochondrial-directed drugs should take in account two major limitations: first, the lack of organ-specificity, leading to a greater accumulation in mitochondria-rich tissues; and second the typically used chemicals tend to accumulate in the matrix and the matrix-facing surface of the inner mitochondrial membrane, over other important mitochondrial compartments (Leitao-Rocha et al., 2015). The majority of mitochondria-targeted compounds were already able to identify and get ahead of these limiting factors in human clinical trials, defining the amount of the compound that can be administered safely. For example, MitoQ, was already developed as a pharmaceutical by Antipodean Pharmaceuticals Inc. (Smith et al., 2008) and clinical trials are running, namely in type 2 diabetic patients, where MitoQ treatment was able to decrease ROS levels and significantly reduced the adhesion of leukocytes to endothelial cells in type 2 diabetic individuals (Escribano-Lopez et al., 2016).

Likewise, approaches designed to target Nox-derived ROS overproduction are also under intense scrutiny. Indeed, the genetic deletion or inhibition of Nox2 seems effective in improving cerebral vessels functioning in aged APP mice (Park et al., 2007, 2008). Apocynin and diphenyleniodonium are widely used to ameliorate Nox-related cerebrovascular dysfunction, however, both compounds present non-specific effects and cannot be used in the clinic (**Table 1**) (De Silva and Miller, 2016). In this line, several attempts are being made to synthesize new Nox inhibitors including fulvene-5, triphenylmethane derivatives, grindelic acid, and ML171 (**Table 1**) (De Silva and Miller, 2016). However, results are scarce and further studies are needed to demonstrate that those compounds are potential therapeutics for the treatment of neurodegenerative conditions characterized by oxidative stress-associated vascular alterations.

In sum, despite the existing information about the role of cerebrovascular oxidative stress in neurodegenerative conditions, a long way is still ahead to clarify the mechanisms of age-associated vascular damage and subsequent neurodegenerative conditions. New information is crucial for the design of more effective therapeutic strategies.

AUTHOR CONTRIBUTIONS

CC performed literature search and wrote the paper. PM provided a critical revision of the paper.

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Sweet Stress: Coping With Vascular Dysfunction in Diabetic Retinopathy

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Oxidative stress plays key roles in the pathogenesis of retinal diseases, such as diabetic retinopathy. Reactive oxygen species (ROS) are increased in the retina in diabetes and the antioxidant defense system is also compromised. Increased ROS stimulate the release of pro-inflammatory cytokines, promoting a chronic low-grade inflammation involving various signaling pathways. An excessive production of ROS can lead to retinal endothelial cell injury, increased microvascular permeability, and recruitment of inflammatory cells at the site of inflammation. Recent studies have started unraveling the complex crosstalk between retinal endothelial cells and neuroglial cells or leukocytes, via both cell-to-cell contact and secretion of cytokines. This crosstalk is essential for the maintenance of the integrity of retinal vascular structure. Under diabetic conditions, an aberrant interaction between endothelial cells and other resident cells of the retina or invading inflammatory cells takes place in the retina. Impairment in the secretion and flow of molecular signals between different cells can compromise the retinal vascular architecture and trigger angiogenesis. In this review, the synergistic contributions of redox-inflammatory processes for endothelial dysfunction in diabetic retinopathy will be examined, with particular attention paid to endothelial cell communication with other retinal cells.

Keywords: diabetic retinopathy, blood–retinal barrier, retinal endothelial cells, oxidative stress, inflammation, apoptosis, neovascularization

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INTRODUCTION

Diabetic retinopathy is a leading cause of visual impairment in the working-age population of the Western world (Cheung et al., 2010). Of an estimated 468 million people with diabetes mellitus worldwide (Ogurtsova et al., 2017), more than 90 million diabetics have some form of diabetic retinopathy, of which one-third has severe vision-threatening diabetic retinopathy (Yau et al., 2012). Due to the increasing prevalence of diabetes, aging of the population, and increased life expectancy of those with diabetes, these numbers are expected to rise.

Abbreviations: AGEs, advanced glycation end products; BRB, blood–retinal barrier; CD18, integrin ligand integrin beta-2; CD40, cluster differentiation 40 receptor; ICAM-1, intercellular adhesion molecule-1; IRMA, intraretinal microvascular abnormalities; IL, interleukin; NF-κB, nuclear factor-κB; NOS, nitric oxide synthase; Nox, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; OCTA, optical coherence tomography angiography; PKC, protein kinase C; RAGE, receptor for AGEs; ROS, reactive oxygen species; SIRT1, sirtuin 1; STAT, signal transducers and activators of transcription proteins; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Diabetic retinopathy is very common among diabetic patients. Almost all patients with type 1 diabetes and more than 60% of patients with type 2 diabetes have some degree of retinopathy after 20 years with diabetes (Fong et al., 2004). In the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), 3.6% of patients with type 1 diabetes and 1.6% of patients with type 2 diabetes were legally blind. In the group of patients with type 1 diabetes, 86% of blindness was attributable to diabetic retinopathy. In the group of type 2 diabetes patients, in which other eye diseases are common, diabetic retinopathy is responsible for one-third of the cases of legal blindness (Klein et al., 1984).

The classification of diabetic retinopathy into stages is based on the presence of visible ophthalmologic changes and the manifestation of retinal neovascularization. A first stage in diabetic retinopathy is preclinical retinopathy, without alterations in the eye fundus. Then, the disease progresses to mild non-proliferative retinopathy that is characterized by the presence of a few microaneurysms. The third stage is moderate non-proliferative retinopathy and it is characterized by the presence of microaneurysms, intraretinal hemorrhages, or venous beading. Microaneurysms and hemorrhages, identified as red dots, are the initial changes seen on ophthalmoscopic examination and fundus photography. The counting of red dots has been suggested as an appropriate marker of retinopathy progression (Klein et al., 1984, 1995). Microaneurysm formation is associated with localized proliferation of endothelial cells, loss of pericytes, and alterations of the capillary basement membrane (Cunha-Vaz, 1978). The fourth stage is severe non-proliferative diabetic retinopathy. In this stage, there is an increase in signs of retinal ischemia, which consists of cotton-wool spots, IRMA, several regions with lack of capillary perfusion, and significant venous beading. IRMAs are abnormal, dilated retinal capillaries that may represent forms of early neovascularization or forms of communication between arterioles and venules in zones of capillary occlusion. This stage is diagnosed using the "4-2-1 rule" of the Early Treatment of Diabetic Retinopathy Study, in which severe non-proliferative diabetic retinopathy is diagnosed if the patient has any of the following: diffuse intraretinal hemorrhages and microaneurysms in 4 quadrants, venous beading in $\geq$2 quadrants, or IRMA in $\geq$1 quadrant. Patients at this stage likely progress to the proliferative stage (Aiello, 2003). Proliferative diabetic retinopathy refers to a severe stage of diabetic retinopathy in which new blood vessels proliferate on the surface of the retina and posterior surface of the vitreous (Wu et al., 2013; Stitt et al., 2016). An important additional category in diabetic retinopathy is diabetic macular edema, which is an important manifestation of diabetic retinopathy and represents the most common cause of vision loss in patients with diabetes (Lee et al., 2015). Diabetic macular edema, characterized by retinal thickening resulting from leaky blood vessels, can develop at all stages of retinopathy, although it is more prevalent during the later phases (Antonetti et al., 2012).

The duration of diabetes is largely associated with the development and progression of retinopathy. The risk of diabetic retinopathy is also attributable to glycated hemoglobin and diabetes duration, as good glycemic control reduces the progression of retinopathy (Stratton et al., 2001; Chew et al., 2014). In addition, other factors may contribute to the risk for developing diabetic retinopathy and disease progression, such as dyslipidemia, high blood pressure, and chronic inflammation (Cardoso et al., 2017).

Several options are available to treat diabetic retinopathy in its later stages, such as laser photocoagulation, intravitreal injections of anti-VEGF or corticosteroids, and surgery. Laser treatment is highly effective in preventing visual loss and preserving vision in patients with advanced diabetic retinopathy and diabetic macular edema, who are at risk if not treated (Deschler et al., 2014). It should be noted that laser photocoagulation is an invasive procedure and vision is rarely improved or restored. Intravitreal therapies with anti-angiogenic agents, such as anti-VEGF, have improved the prognosis for diabetic macular edema (Schmidt-Erfurth et al., 2017) and anti-VEGF treatment is now the first-line therapy for diabetic macular edema involving the central macula (Thomas et al., 2013). The use of corticosteroids for treating patients with diabetic macular edema is suggested as a second choice, particularly for patients not responding to anti-VEGF therapy (Schmidt-Erfurth et al., 2017). Vitreous hemorrhage is a frequent complication of diabetic retinopathy, and vitreoretinal surgery is indicated for cases of non-clearing vitreous hemorrhage from proliferative diabetic retinopathy in patients who did not have previous laser photocoagulation (Berrocal et al., 2016). Pars plana vitrectomy, the surgical technique usually employed, has been suggested as a potential treatment option for diabetic macular edema patients with vitreoretinal traction, although the procedure remains controversial in cases without traction.

The pathogenesis of the disease is complex and several vascular, inflammatory, and neuronal mechanisms are known to be involved. The hallmark of diabetic retinopathy is the dysfunction of the inner BRB leading to increased permeability of serum constituents that may gain access to neural tissue. Genetic, metabolic, and environmental factors contribute to the four known biochemical pathophysiological pathways: polyol, AGEs, hexamine, and PKC. The alterations in these mechanisms can lead to hypoxia, oxidative stress, and inflammation that contribute to the breakdown of the BRB.

MICROVASCULAR ALTERATIONS IN DIABETIC RETINOPATHY

Historically, diabetic retinopathy has been thought to primarily affect the microvasculature (Lynch and Abramoff, 2017) probably due to the fact that the mainstay tests for screening for diabetic retinopathy predominantly detect microvascular changes. Besides the presence of microaneurysms and soft exudates, macular edema, and neovascularization (which are visible by ophthalmoscopy or fundus photography), it is now known that other retinal cell types are affected by diabetes and may compromise or contribute to visual impairment. In fact, diabetic retinopathy is characterized by progressive changes in the retinal microvasculature with accompanying neuroglial damage (Fletcher et al., 2007). The blood supply to the inner

retina is composed of three different vascular plexuses embedded in the neural tissue: a layer of vessels that lies within the nerve fiber layer with branches extending into the ganglion cell layer, and two layers lying along each side of the inner nuclear layer (**Figure 1**). A recent study using OCTA, a non-invasive method of 3D imaging of the retinal and choroidal circulations, has revealed a more detailed vascular anatomy of human retina than previously described (Campbell et al., 2017). With a projection-resolved OCTA algorithm, an improved system of OCTA segmentation boundaries has been identified, with two complexes and four distinct vascular plexuses in the retina. The identification in the macula of an intermediate capillary plexus and deep capillary plexus at the level of deep vascular complexes may be useful in the assessment of vascular changes, such as capillary dropout, as well as abnormal vessels in all retinal layers in diabetic retinopathy (Campbell et al., 2017). This approach can provide earlier identification of capillary layers that are more affected in this and other retinal diseases, contributing to a deeper understanding of retinal pathophysiology.

The retinal vascular endothelium is a monolayer of cells covering the vascular lumen that supplies and drains the inner retina. On the one hand, the endothelium acts as a selective barrier (inner BRB) between the blood circulation and the neural retina. The presence of tight junctions prevents the outward flow of macromolecules and maintains the local microenvironment. On the other hand, the endothelium efficiently supplies oxygen and nutrients to the neural retina.

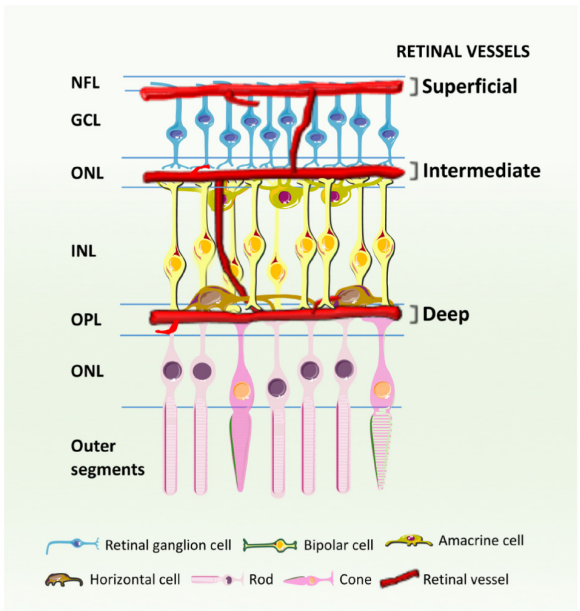

FIGURE 1 | Schematic cross-section showing the retinal blood vessels lining the inner surface of the retina. Three capillary plexuses are embedded among retinal neurons: the superficial layer lies within the inner nerve fiber layer (NFL) with branches extending into the ganglion cell layer (GCL), while the intermediate and deep capillary plexuses align along each sides of the inner nuclear layer (INL). ONL, outer nuclear layer. IPL, inner nuclear layer; OPL, outer plexiform layer.

However, under certain conditions, such as hyperglycemia, retinal endothelial cells become particularly vulnerable. Plasma glucose is transported across the cell membrane through facilitative glucose transporters, namely the constitutive isoform GLUT1 (Badr et al., 2000; Fernandes et al., 2003, 2004). At present, there is still conflicting data on GLUT1 protein regulation under high glucose conditions. Despite a localized increase in GLUT1 on the endothelial cells in more than half of the post-mortem retinas from patients with long-standing diabetes (>17 years) (Kumagai et al., 1996), no changes were observed in an animal model of long-standing diabetes (Fernandes et al., 2003), both using immunogold staining. However, a downregulation in total retinal GLUT1 and retinal microvascular GLUT1 was reported in alloxan- and streptozotocin-induced diabetic rats (Balabanov and Dore-Duffy, 1998; Badr et al., 2000; Fernandes et al., 2004). These contradictory results may be due to different species and duration of disease. Furthermore, in addition to GLUT1, studies on the contribution of other GLUT isoforms present in the BRB may provide a better understanding of retinal metabolism in diabetic retinopathy.

Most cells of the body when exposed to hyperglycemia are able to maintain their intracellular glucose levels relatively constant by regulating its transport into the cell. In contrast, endothelial cells struggle to control their intracellular basal glucose levels, and its increase can alter numerous cellular functions, leading to cell damage (Du et al., 2003; Kowluru and Abbas, 2003). Thus, diabetes damages endothelial cells and triggers a cascade of signaling events that leads to metabolic and biochemical derangements.

A unifying mechanism of hyperglycemia-induced endothelial cellular injury was proposed by Brownlee (2005), in which the overproduction of superoxide anions by the mitochondrial electron transport chain during the process of oxidative phosphorylation is the common upstream event that may stimulate diverse biochemical pathways known to play a role in the pathogenesis of diabetic retinopathy: (i) increased flux through the polyol pathway and activation of aldose reductase (Lorenzi, 2007); (ii) enhanced non-enzymatic glycation; (iii) increased production of diacylglycerol; and (iv) stimulation of PKC (**Figure 2**). The overall effects of these mechanisms are an increase in ROS production and stimulation of the oxidative stress (Brownlee, 2005; Wu et al., 2014), resulting in a range of biochemical and metabolic abnormalities.

The overproduction of superoxide anion by the mitochondria under hyperglycemic conditions can leave an early imprint in cells of the vasculature, enabling the development of microvascular abnormalities even when good glycemic control is achieved. Superoxide anion has been suggested as a key player in this metabolic memory effect, and potential mechanisms for propagating this memory are the increased accumulation of potentially harmful agents, such as AGEs, which leads to glycation of mitochondrial proteins, lipids, and nucleic acids (Berezin, 2016). These injurious effects are not easily reversed after a reinstatement of good glucose control on hyperglycemia-induced oxidative stress, mainly if poor glycemic control is maintained for longer durations, and some alterations may be

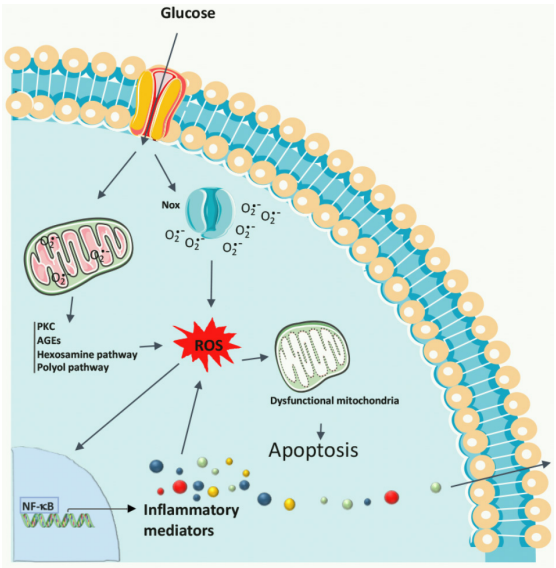

FIGURE 2 | Biochemical pathways leading to diabetic retinopathy. Chronic hyperglycemia promotes the increase in ROS formation, originating in the mitochondria or produced by Nox, and also activates many metabolic pathways, including PKC, polyol pathway, AGEs, and the hexosamine pathway, as well as the production of inflammatory mediators. Oxidative stress damages mitochondrial DNA resulting in a decline in mitochondrial function, which accelerates cell apoptosis, contributing to the development of diabetic retinopathy.

even permanent because of epigenetic changes (Ceriello, 2009). In fact, AGEs have been correlated with presence of varying stages of diabetic retinopathy, independent of HbA1c (Genuth et al., 2015). Furthermore, hyperglycemia-induced superoxide production, mainly from mitochondria, may lead to increased PKC activity and chronic low-grade inflammation mediated by activation of NF-κB. PKC activation leads to increased ROS generation via activation of mitochondrially localized adaptor protein Shc ($p66^{Shc}$, which functions as a redox enzyme implicated in mitochondrial ROS generation and translation of oxidative signals into apoptosis) and NADPH oxidase signaling. Epigenetic changes, such as DNA methylation and acetylation and post-translational histone modifications, are responsible for persistent $p66^{Shc}$ overexpression, promoting the metabolic memory and participating in diabetic retinopathy progression. Transient episodes of hyperglycemia-induced ROS formation in endothelial cells are sufficient to induce epigenetic changes responsible for perpetuating the metabolic memory by inducing sustained increase in the NF-kB subunit p65 gene expression and in the expression of p65-dependent pro-inflammatory genes (Perrone et al., 2014; Berezin, 2016). Despite the dysfunction of retinal mitochondria and impairment of the metabolic pathways (Sone et al., 1996), subclinical inflammation, dysregulation in cell–cell communication, and maintenance of cell homeostasis are also associated with early diabetes-related endothelial dysfunction.

Histological analyses of diabetic retinas have shown that loss of the retinal capillary pericytes (smooth muscle cells that provide tone to the vessels) and endothelial cells are two invariable features of the earliest events of diabetic retinopathy (Durham and Herman, 2011). Pericyte dropout, manifested by their degeneration (ghost cells), may eventually lead to the formation of acellular capillaries, microaneurysms with decreased retinal perfusion, capillary basement thickening, and induction of a series of biochemical and metabolic alterations (Barot et al., 2013). Non-vascular cells, such as Müller cells and other glial cells, also undergo apoptosis. Capillary occlusion and retinal ischemia cause increased expression and release of a number of growth factors and cytokines. These factors include VEGF, which increases vascular permeability and favors the growth of abnormal new vessels (Frank, 2004). In proliferative diabetic retinopathy, pathological angiogenesis driven by VEGF originating from pericytes, retinal ganglion cells, and glia has been implicated in irreversible vision loss (Frank, 2004). In fact, VEGF levels are significantly increased in both vitreous and aqueous fluids of patients with proliferative diabetic retinopathy compared with samples from patients with non-proliferative diabetic retinopathy or without diabetes (Aiello et al., 1994; Duh and Aiello, 1999). However, low levels of the pro-angiogenic factor VEGF in the presence of increased levels of angiopoietin-2 were associated with activation of apoptotic pathways, detrimental to survival and proliferation pathways, leading to endothelial cell death and vessel regression in the early stages of diabetic retinopathy (Bento et al., 2010). VEGF is likely to be part of the angiogenic paradox of diabetes, in which both pro- and anti-angiogenic conditions may coexist with chronic hyperglycemia, and in particular, in the time course of retinopathy.

Other agents are also involved in the development of pathological angiogenesis and include angiopoietin-1 and -2 (Joussen et al., 2002b; Hammes et al., 2004; Cai et al., 2008), erythropoietin (Chen et al., 2009; Hernandez and Simo, 2012), the renin–angiotensin–aldosterone system (Wilkinson-Berka et al., 2007), fibroblast growth factor 2, TNF, and stromal-derived factor 1 alpha and its receptor, CXCR4. Many of these factors act in concert to mediate the key steps leading to angiogenesis, including protease production, endothelial cell proliferation, migration, and tube formation (Grant et al., 2004). Furthermore, the balance between angiogenic and anti-angiogenic factors is a key factor in determining the development and progression of proliferative diabetic retinopathy (Simo et al., 2006; Lima e Silva et al., 2007).

REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS IN RETINAL ENDOTHELIAL CELLS DURING DIABETES

Glucose oxidation, high oxygen demand, and the presence of a high content in polyunsaturated fatty acids render the retina particularly susceptible to oxidative stress (Anderson et al., 1984). Under physiological conditions, ROS are necessary to support a variety of cellular processes, such as metabolism,

proliferation, differentiation, immune system regulation, and vascular remodeling (Wilkinson-Berka et al., 2013). However, disturbances in the balance between the generation and rapid scavenging of ROS generate toxic effects that can result in cell death (Calabrese et al., 2007). Superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical (•OH) are considered the primary ROS, since they are unstable and react with biological molecules, such as proteins, lipids, carbohydrates, and nucleic acids.

Increased oxidative stress is detected in the retinas of experimental animal models with diabetes and galactosemia (Barot et al., 2011; Fernandes et al., 2014). The primary sources of intracellular ROS are the mitochondrial electron transport chain, cytochrome P450, xanthine oxidase, NOS, and Nox (Bedard et al., 2007; Millar et al., 2007; Forstermann, 2008; Sorce et al., 2012; Takac et al., 2012; Wilkinson-Berka et al., 2013). Elevated levels of ROS can be found as a result of the compromised activity of ROS-detoxifying systems, such as reduced glutathione, vitamin E, superoxide dismutase, catalase, thioredoxin peroxidase, and heme oxygenase (Wilkinson-Berka et al., 2013).

In retinal endothelial cells, high glucose levels can lead to overproduction of mitochondrial ROS, which then activate the poly-ADP-ribose polymerase (PARP) pathway decreasing the activity of glyceraldehyde 3-phosphate dehydrogenase and the levels of sirtuin 1 (SIRT1, a class III histone deacetylase) (Zheng et al., 2012). Interestingly, SIRT1 downregulation that leads to sustained responses of inflammatory and apoptotic proteins, such as NF-κB, Bax, and PARP, was maintained and propagated in cultured retinal endothelial cells, even after 2 weeks of normal glucose following 1 week of exposure to high glucose concentrations (Zheng et al., 2012). A potential mechanism for propagating cellular metabolic memory linked to high glucose exposure involves an excess of ROS production from mitochondria, and SIRT1 has been identified as a key player in this memory of retinal endothelial cells (Ihnat et al., 2007; Zheng et al., 2012).

A growing body of evidence indicates that Nox enzymes play an important role in the development of diabetic retinopathy (Li et al., 2010; Rojas et al., 2013; Kowluru et al., 2014; Wang et al., 2014). The superoxide-generating Nox enzymes are a primary source of extra-mitochondrial ROS generation (Jiang et al., 2011; Rinnerthaler et al., 2012). Besides their major role in the production of ROS (mainly superoxide anion and hydrogen peroxide), Nox isoforms have pro-inflammatory effects in the retina. Nox1, Nox2, and Nox4 are three of seven isoforms (Nox1 to -5, Duox1, and Duox2) that are expressed in retinal endothelial cells (Li et al., 2010) and all these isoforms can stimulate endothelial cell proliferation and tubulogenesis. *In vitro* studies with retinal endothelial cells under hypoxic and high glucose conditions revealed an upregulation of mRNA expression and protein levels of Nox4, ROS generation, and VEGF levels. Inhibition of Nox4 activity by statins (lovostatin) downregulates hypoxia-inducible factor 1-alpha and STAT3-mediated VEGF expression and ameliorate retinal vascular leakage in diabetic retinopathy (Li et al., 2010). GKT137831 (member of the pyrazolopyridine dione family), a dual inhibitor of Nox1 and Nox4, reduced the increased gene and protein expression of VEGF, monocyte chemoattractant protein-1, and leukocyte adhesion molecules as well as vascular leakage in an experimental model of ischemic retina (Deliyanti and Wilkinson-Berka, 2015). These findings imply an important role of Nox1/4 in endothelial function via regulation of migration and infiltration of monocytes/macrophages and BRB breakdown. Among the three isoforms, Nox2 has been the widest studied since its role in phagocytic defense and inflammation in diabetic retinopathy has been well established. In fact, increased levels of Nox2 in retinal blood vessels were associated with increased oxidative stress in the retina in an experimental model of diabetic retinopathy. Deletion of Nox2 or apocynin (a selective Nox inhibitor) treatment prevented diabetes-induced increases in ROS and ICAM-1 levels as well as retinal leukostasis and vascular leakage, suggesting that Nox2 is a key player in pathological conditions characterized by retinal vascular inflammatory reactions (Al-Shabrawey et al., 2008). Additionally, hyperglycemia-induced endothelial damage can generate reactive nitrogen species, such as peroxynitrite ($ONOO^-$), through the rapid reaction of superoxide anion with nitric oxide. Peroxynitrite is a highly potent oxidant and nitrosylating agent that promotes leukocyte adhesion to retinal vessels and induces BRB breakdown (Leal et al., 2007; Pacher et al., 2007; Goncalves et al., 2012).

INFLAMMATION IN DIABETIC RETINOPATHY

Diabetic retinopathy has been recognized as chronic inflammatory disease, and local inflammation has been indicated as a novel risk factor for its development and progression (Lee et al., 2015; Atchison and Barkmeier, 2016). The origin of the inflammatory environment in the retina during diabetes still needs clarification. Nevertheless, since retinal apoptotic cell death occurs in diabetic conditions that may trigger an inflammatory condition, some authors have proposed that the metabolic alterations are at the genesis of inflammation (Tang and Kern, 2011).

Inflammatory cytokines have a role in the pathophysiology of this disease. Inflammatory cytokines, such TNF, IL-6, and C-reactive protein, mainly produced by adipose tissue and macrophages, have been detected in the serum of type 2 diabetic patients (Ellulu et al., 2017) and were associated with the microvascular complications of diabetic retinopathy (Schram et al., 2005). However, local inflammation seems to be more relevant for the development of diabetic retinopathy. Several cytokines, chemokines, and other factors are increased in the retina and vitreous of diabetic patients and animal models of diabetes (Hernandez et al., 2005; Tang and Kern, 2011; Abcouwer, 2013).

Inflammation mediates molecular and structural alterations associated with diabetic retinopathy, such as the breakdown of the BRB. Inflammation is the basis for the treatment with corticosteroids. Glucocorticoids decrease the inflammatory processes and improve BRB function by inhibiting leukocyte recruitment (Tamura et al., 2005). Inflammation also plays a role in the development of diabetic macular edema due to the accumulation of leukocytes on the surface of capillaries, an early

event in BRB breakdown (Leal et al., 2007). Interestingly, vitreous VEGF levels are not increased in all patients with proliferative diabetic retinopathy (Aiello et al., 1994), which may be the reason why some patients do not respond to anti-VEGF therapies (Singer et al., 2016). It has been suggested that in such non-VEGF responders, intravitreal steroid injection would be more appropriate because pro-inflammatory cytokines probably play a major pathological role (Corcostegui et al., 2017).

LINKING INFLAMMATION AND OXIDATIVE STRESS IN DIABETIC RETINOPATHY

Chronic hyperglycemia-associated oxidative stress and low-grade inflammation are considered to play critical roles in the onset and progression of the complications of diabetes, including diabetic retinopathy (Xu et al., 2009; Frey and Antonetti, 2011). The mechanisms by which oxidative stress triggers inflammation and inflammation induces oxidative stress have not been totally clarified. Oxidative stress increases the production of cytokines through the activation of the transcription factors, such as NF-κB, STAT, and activator protein-1 that lead to the transcription of genes encoding cytokines (Turpaev, 2002; Wilkinson-Berka et al., 2013). The activity of NF-κB has been demonstrated to be increased in endothelial cells, pericytes, and glial cells in experimental models of diabetic retinopathy, and in the retinas of diabetic patients (Romeo et al., 2002; Kowluru et al., 2003; Harada et al., 2006). The inhibition of NF-κB inhibits diabetes-induced production of inflammatory molecules in retinal tissue, highlighting the essential role of NF-κB in diabetes-induced retinal inflammation (Nagai et al., 2007).

Cytokines are also able to increase the production of ROS. TNF, IL-1β, and interferon-γ induce ROS production in retinal pigment epithelial cells. TNF and IL-1β increase ROS production via mitochondria and Nox, respectively. Interferon-γ elicits ROS production via mitochondria and Nox (Yang et al., 2007). The production of chemokine (C-C motif) ligand 2 during retinal vascular inflammation requires activation of the Nox pathway through NF-κB and Akt signaling pathways (Zhang W. et al., 2009). A convincing report further confirmed that cytokines trigger ROS production in the retina by demonstrating that IL-1β administration to the vitreous of experimental animals increases oxidative stress in the retina, similar to studies done in diabetes models (Kowluru and Odenbach, 2004a,b). Pro-inflammatory cytokines induce the formation of ROS and NO, which, through the formation of peroxynitrite, reduce the content of small heat shock protein 27 and lead to apoptotic cell death of retinal endothelial cells (Nahomi et al., 2014).

CROSSTALK BETWEEN ENDOTHELIAL CELLS AND CIRCULATING MEDIATORS AND INFLAMMATORY CELLS

Endothelial cells together with the regulatory pericyte and glial cells surrounding the retinal vessels are essential for maintaining BRB integrity and normal function. In diabetic patients and experimentally induced diabetic animals, death of pericytes and endothelial cells has been reported, and precedes histological evidence of retinopathy (Mizutani et al., 1996). Altered permeability of retinal capillaries that leads to the breakdown of the inner BRB is considered a hallmark of diabetic retinopathy (Klaassen et al., 2013). However, the molecular mechanisms underlying altered permeability of retinal endothelial cells remain to be clarified. Mounting evidence suggests that inflammation plays an important role in the development of diabetic retinopathy (Tang and Kern, 2011). In fact, elevated levels of pro-inflammatory cytokines have been detected in the vitreous of diabetic patients with retinopathy (Abu el Asrar et al., 1992) and in diabetic rat retinas with increased vascular permeability (Carmo et al., 2000; Joussen et al., 2002a).

It was demonstrated that TNF has an important role in the pathogenesis of diabetic retinopathy, being an important inducer of endothelial cell apoptosis (Robaye et al., 1991; Polunovsky et al., 1994). TNF also increases retinal endothelial cell monolayer permeability by inducing a decrease in the tight junction proteins zonula occludens (ZO)-1 and claudin-5 and a localization shift of both proteins (Aveleira et al., 2010). However, an apparent contradiction arose regarding if the presence of TNF is more important for BRB breakdown in the initial or late phases of diabetic retinopathy (Aveleira et al., 2010; Huang et al., 2011). Using a TNF-knockout mouse, it was observed that the absence of TNF had no effect in the initial phases of diabetic retinopathy-associated BRB breakdown, even though it prevented retinal leukostasis. At later phases, BRB breakdown was suppressed by the absence of TNF (Huang et al., 2011). Moreover, it was demonstrated that high glucose-induced cell death is due to the activation of TNF receptor 1 by TNF. Thus, blocking the activity of this receptor could be an adequate strategy to avoid cell loss in diabetic conditions (Costa et al., 2012). Additionally, TNF could be a good therapeutic target for the prevention of diabetic retinopathy progression (Zhang et al., 2011). Furthermore, it has been suggested that its concentration in the tear fluid could be used as a biomarker for the detection of diabetic retinopathy (Costagliola et al., 2013).

The cytokine IL-1β is increased in the vitreous of patients with diabetic retinopathy and has been implicated in disease progression (Mao and Yan, 2014). It was suggested that IL-1β contributes to accelerated apoptosis of retinal endothelial cells under hyperglycemic conditions (Kowluru and Odenbach, 2004b). In fact, hyperglycemia triggers the increase in IL-1β production in diabetic retinopathy that is mainly produced by retinal vascular endothelial cells (Liu et al., 2012). IL-1β itself induces an autostimulation in endothelial cells, which is the main mechanism for sustained IL-1β overexpression (Liu et al., 2012). It was pointed out that interrupting the IL-1β-induced autostimulation could limit the progression of diabetic retinopathy. IL-1β-induced autostimulation occurs via forkhead transcription factor (FoxO1) upregulation in endothelial cells, and silencing FoxO1 reduces the IL-1β expression levels (Wu et al., 2017).

When inflammation persists chronically, leukocytes that are recruited to the inflamed tissue can induce tissue damage by

prolonged secretion of chemical mediators and toxic ROS (Noda et al., 2012), further contributing to the progression of diabetic retinopathy. In fact, during diabetes, leukocytes have been shown to be activated (Miyamoto et al., 1998; Kinukawa et al., 1999; Nonaka et al., 2000) and to become a source of Nox-derived ROS (Mohanty et al., 2000; Wong et al., 2002). Leukocytes, therefore, play a critical role in capillary degeneration and permeability and contribute to retinal edema, ischemia, and angiogenesis (Ishida et al., 2004). It was observed that leukocytes colocalize with dying endothelial cells in the diabetic retina (Schroder et al., 1991). Moreover, leukocytes from diabetic mice trigger the death of more retinal endothelial cells than leukocytes from non-diabetic mice (Li et al., 2012), and neutrophils from diabetic animals exhibit higher levels of surface integrin expression and integrin-mediated adhesion (Barouch et al., 2000). Also, leukocytes from diabetic patients were found to induce the death of human retinal endothelial cells, by a mechanism that is dependent on direct contact and indicates that cell surface molecules are involved (Tian et al., 2013). Blocking integrin ligand integrin beta-2 (CD18) on leukocytes or ICAM-1 on the surface of retinal endothelial cells inhibits the leukocyte-mediated death of endothelial cells (Barouch et al., 2000; Tian et al., 2013). Thus, therapies that block inflammation, as well as leukocyte–endothelial cell–cell interactions can be envisaged as potential treatments for diabetic retinopathy. In fact, in diabetic CD18- and ICAM-1-deficient mice there are fewer adherent leukocytes in the retinal vasculature, which is associated with fewer damaged endothelial cells and reduced vascular leakage (Joussen et al., 2004). The administration of berberine, which has anti-inflammatory and antioxidant properties, inhibits leukocyte adhesion to retinal endothelial cells, as well as the leukocyte-mediated death of endothelial cells by the downregulation of ICAM-1 and CD18 (Tian et al., 2013).

Calcium dobesilate has been prescribed for the treatment of diabetic retinopathy (Garay et al., 2005), reducing the progression of the disease (Zhang et al., 2015). In an animal model of diabetes, we reported that calcium dobesilate prevents the BRB breakdown induced by diabetes by preventing the alterations in the tight junction proteins and decreasing leukocyte adhesion to retinal vessels (Leal et al., 2010). The beneficial properties of calcium dobesilate partly rely on the decrease of pro-inflammatory processes and oxidative stress in the retina (Voabil et al., 2017). Sitagliptin, an oral anti-hyperglycemic drug of the dipeptidyl peptidase-4 inhibitor class (Drucker and Nauck, 2006), also prevents the breakdown of BRB, inflammation, apoptosis, and alterations in the tight junction complexes in an animal model of diabetes (Goncalves et al., 2014).

CROSSTALK BETWEEN ENDOTHELIAL AND RETINAL CELLS

Pericytes

The loss of pericytes is an early histopathological hallmark of diabetic retinopathy (Curtis et al., 2009). These cells have an important role in the maintenance of microvascular homeostasis, since they are involved in the maintenance of capillary tone and in the regulation of the vessel growth. In addition, pericytes also preserve the prostacyclin-producing ability and protect against lipid-peroxide-induced injury of the endothelial cells (Bergers and Song, 2005; Hamilton et al., 2010). Co-culture experiments have suggested that pericytes inhibit endothelial cell proliferation in a contact- or proximity-dependent manner (Orlidge and D'Amore, 1987). Also, pericytes increase the barrier properties of endothelial cell monolayers (Balabanov and Dore-Duffy, 1998), through transforming growth factor-beta (Dohgu et al., 2005) production. Pericytes provide paracrine signals that promote vessel stabilization. A pericyte-derived angiopoietin-1 multimeric complex binds to the Tie2 receptor on endothelial cells, inducing the expression of the tight junction protein occludin (Hori et al., 2004) *in vitro*. Therefore, the loss of pericytes could predispose the vessels to angiogenesis, thrombogenesis, and endothelial cell injury, leading to the clinical manifestations of diabetic retinopathy. Retinal pericytes accumulate AGEs during diabetes, altering their function and decreasing cell survival with harmful consequences to the BRB, leading to the progression of diabetic retinopathy (**Figure 3**). Indeed, the interaction between AGEs and their receptors (known as RAGEs) elicits ROS generation in cultured retinal pericytes, thereby inducing apoptotic cell death (Yamagishi et al., 2005). AGEs induce NF-κB activation, decrease the ratio of Bcl-2/Bax, and subsequently increase the activity of caspase-3, a key enzyme in the apoptosis of pericytes. Moreover, AGEs upregulate RAGEs expression levels in pericytes via ROS production (Yamagishi, 2011). These positive feedback loops transduce the AGE signals, thus further exacerbating their cytotoxic effect. Interestingly, it has been suggested that the loss of pericytes does not directly influence BRB barrier properties but, on the other hand, sensitizes endothelial cells to the effects of VEGF-A and angiopoietin-2, thus leading to changes in vascular permeability and angiogenesis (Park et al., 2014, 2017). In the absence of pericytes, the endothelial cells lose their regulation of proliferation and form new vessels (Beltramo and Porta, 2013). Therefore, the crosstalk between pericytes and endothelial cells is essential for vascular function and retinal homeostasis.

Astrocytes

Astrocytes are glial cells present in the retina enveloping ganglion cell axons and forming axonal and vascular glial sheaths. These cells are active part of the BRB, sensing alterations in neuronal function and controlling the transfer of components from blood vessels to neurons. In fact, astrocytes modulate retinal homeostasis by nourishing neurons with glucose and by regulating extracellular potassium levels and metabolism of neurotransmitters. Although less abundant in retinal tissue than Müller cells, astrocytes play a crucial role in the transmission of retinal signals by retinal ganglion cells to the superior colliculus in the brain. In response to noxious stimuli, astrocytes suffer astrogliosis, a process characterized by changes in morphology, an increase in their proliferation rate, and the expression of glial fibrillary acidic protein (de Hoz et al., 2016) (**Figure 3**). In high glucose conditions, astrocytes proliferate and change their motility and distribution due to an increase in the expression of adhesion molecules (Shin et al., 2014). In addition,

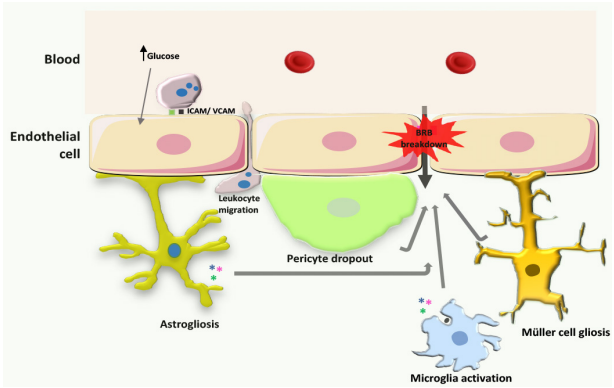

FIGURE 3 | Processes leading to BRB breakdown in diabetic retinopathy. During the development of diabetic retinopathy, increased production of ROS leads to increased expression of intercellular adhesion molecules in endothelial cells, and consequently increased leukocyte adhesion. Concurrently, astrocytes, Müller cells, and microglia become activated, promoting cytokine release, and pericytes surrounding endothelial cells die by apoptosis. Moreover, vascular ROS and inflammatory mediators that are released by inflammatory cells affect endothelial tight junctions leading to increased BRB permeability.

in diabetic retinopathy, astrocytes increase the expression of NF-κB (Shin et al., 2014) and increase the inflammatory milieu with direct consequences on the properties of the BRB (Barber et al., 2000). Also, the increase in angiopoietin-2 produced by endothelial cells in the diabetic retina (Yao et al., 2007) induces astrocyte apoptosis and BRB impairment (Yun et al., 2016).

Müller Cells

Müller cells are the most abundant glial cells in the retina and constitute an anatomic and functional link between neurons and blood vessels, spreading from the subretinal space to the vitreous body. Therefore, Müller cells play a very important role in the nourishment of retinal neurons through their direct link with blood vessels. In addition, by sensing alterations in neural activity, Müller cells are able to buffer the levels of neurotransmitters such as glutamate and ions like potassium and also dispose of metabolic waste derived from the activity of photoreceptors and other retinal neurons. Furthermore, Müller cells regulate vascular tone and have a structural function in the integrity of the BRB (Tout et al., 1993). Alterations in retinal homeostasis lead to Müller cell gliosis, a process characterized by a shift in the supportive role of Müller cells toward a pro-inflammatory state with loss of function. In the diabetic retina, there is an imbalance in glutamate homeostasis (Santiago et al., 2006, 2008, 2009), which may be due to decreased uptake by Müller cells caused by an impairment in glutamate-aspartate transporter and glutamine synthetase activity. The decreased function of glutamate-aspartate transporter and glutamine synthetase lead to an extracellular accumulation of glutamate and lack of glutamine needed for the synthesis of gamma-Aminobutyric acid and glutamate. The accumulation of extracellular glutamate induces neurotoxicity and oxidative stress in retinal neurons culminating in irreversible neural cell loss. Besides their role in glutamate excitotoxicity in diabetic retinopathy, Müller cells also contribute to the progression of the disease by decreasing their potassium buffering capacity. In diabetic patients, the expression of potassium channels is altered in Müller cells due to the accumulation of AGEs, thus decreasing the conductance and increasing the resistance to signal transduction. The alterations in potassium homeostasis lead to neuronal dysfunction and subsequent glutamate excitotoxicity. Activation of CD40, a member of TNF receptor superfamily, induces ATP release in Müller cells and promotes the expression of ATP receptor $P2X_7$ in endothelial cells. These concerted actions lead to the activation of programmed cell death in endothelial cells. The deleterious effects mediated by the activation of CD40 are necessarily triggered by Müller cells, as the activation of CD40 receptor directly in endothelial cells does not impact cell viability (Portillo et al., 2016). Besides ATP, activation of CD40 receptor in Müller cells induces the expression of iNOS, TNF, and IL-1β in microglia/macrophages, promotes retinal leukostasis, and increases protein nitration (Portillo et al., 2014, 2017). Nevertheless, in an *in vitro* model, Müller cells do not induce leukocyte deposition in endothelial cells, indicating that the involvement of Müller cells to BRB breakdown in diabetes is not due to leukocyte adhesion but due to alterations of tight junction protein expression and organization (Leal et al., 2008). Müller cells also increase VEGF expression and contribute to vessel permeability through oxidative stress and the impairment of tight junction proteins, such as occludin, claudin-5, and ZO-1 (Wang et al., 2010) (**Figure 3**).

Microglia

Microglia are the immune cells responsible for maintaining homeostasis in the brain and retina (Du et al., 2017). Under basal conditions, microglia constantly surveil the retinal parenchyma, secreting trophic factors and phagocytizing cell debris and unneeded or dysfunctional synapses (Langmann, 2007; Madeira et al., 2015). Upon alterations in retinal function, microglial cells, in an attempt to restore homeostasis, alter their phenotype. In these circumstances, microglial cells become amoeboid, have high migratory capacity and increased phagocytic efficiency, and release pro-inflammatory factors (Langmann, 2007; Karlstetter et al., 2010; Madeira et al., 2015; Goncalves et al., 2016). The initial low-grade inflammatory profile of microglia is protective for retinal tissue by limiting the damage caused by dysfunctional neurons and endothelial and glial cells. Nevertheless, in chronic retinal diseases the sustained reactivity of microglia becomes deleterious, initiating a cascade of inflammatory events that culminate in neurodegeneration. Several reports have demonstrated the role of microglia in the loss of retinal ganglion cells and endothelial dysfunction with subsequent BRB breakdown in diabetic retinopathy (Krady et al., 2005; Gaucher et al., 2007; Zeng et al., 2008; Grigsby et al., 2014; Goncalves et al., 2016; Sorrentino et al., 2016) (**Figure 3**). Upon exposure to high glucose levels, microglia become reactive and secret pro-inflammatory mediators namely, TNF (Costa et al., 2012)

and IL-1β (Krady et al., 2005; Baptista et al., 2017). These cytokines induce the dysfunction of the BRB by enhancing caspase-3 activity in endothelial cells. In the human diabetic retina, microglia become reactive and strategically distributed along vessels with altered integrity (Zeng et al., 2008; Zhang H. et al., 2009). Interestingly, in proliferative diabetic retinopathy microglia are found in the vicinity of intraretinal hemorrhages, microaneurysms, and cotton-wool spots (Zeng et al., 2008). In addition, microglia directly respond to AGEs, initiating a signaling cascade that summons leukocyte invasion to the neural retina (Grigsby et al., 2014). Evidence shows that microglia become readily activated in response to various stimuli. Activated microglia produce many cytotoxic factors, and Nox-mediated release of superoxide is a common feature in many degenerative diseases (Gao et al., 2002; Qin et al., 2002; Zhang et al., 2005; Zeng et al., 2014). Moreover, there are several reports supporting that microglia-derived ROS may be a crucial and prevailing factor of microglia activation (Gao et al., 2002; Qin et al., 2002; Block, 2008). Nox2-generated ROS in microglia is a key mediator of hypoxia-induced neovascularization (Chan et al., 2013; Zeng et al., 2014). Furthermore, a specific Nox1/4 inhibitor, GKT137831, was able to decrease ischemia-induced ROS production and a pro-inflammatory phenotype in microglia (Deliyanti and Wilkinson-Berka, 2015). Whether Nox could be a valuable therapeutic target in the management of neovascularization and inflammation in diabetic retinopathy has not yet properly addressed.

CONCLUSION

Diabetic retinopathy is a multifactorial disease with a complex pathophysiology, in which excessive production of ROS and oxidative stress play a crucial role in the onset and progression of this disease. Crosstalk between oxidative stress-related and inflammatory pathways is likely to be important in inducing BRB breakdown and pathological neovascularization. A better understanding of endothelial dysfunction and the elucidation of the complex crosstalk between retinal endothelial cells and neuroglial cells and leukocytes, via both cell-to-cell contact and secretion of cytokines may facilitate the development of novel therapies with significant clinical benefit in comparison with existing therapies, namely for neovascularization reduction, but also for the initial stages of the disease. Simultaneous interventions addressing multiple metabolic and signaling pathways may be beneficial to attenuate oxidative stress and inflammation and ameliorate the retinal injury.

AUTHOR CONTRIBUTIONS

AS, RB, IA, and RF wrote the first draft of the manuscript. AA revised the manuscript. All authors revised and approved the final version of the manuscript.

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REVIEW
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Therapeutic Options Targeting Oxidative Stress, Mitochondrial Dysfunction and Inflammation to Hinder the Progression of Vascular Complications of Diabetes

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Type 2 diabetes mellitus is a leading cause of morbidity and mortality worldwide, given its serious associated complications. Despite constant efforts and intensive research, an effective, ubiquitous treatment still eludes the scientific community. As such, the identification of novel avenues of research is key to the potential discovery of this evasive "silver bullet." We focus on this review on the matter of diabetic injury to endothelial tissue and some of the pivotal underlying mechanisms, including hyperglycemia and hyperlipidemia evoked oxidative stress and inflammation. In this sense, we revisited the most promising therapeutic interventions (both non-pharmacological and antidiabetic drugs) targeting oxidative stress and inflammation to hinder progression of vascular complications of diabetes. This review article gives particular attention to the relevance of mitochondrial function, an often ignored and understudied organelle in the vascular endothelium. We highlight the importance of mitochondrial function and number homeostasis in diabetic conditions and discuss the work conducted to address the aforementioned issue by the use of various therapeutic strategies. We explore here the functional, biochemical and bioenergetic alterations provoked by hyperglycemia in the endothelium, from elevated oxidative stress to inflammation and cell death, as well as loss of tissue function. Furthermore, we synthetize the literature regarding the current and promising approaches into dealing with these alterations. We discuss how known agents and therapeutic behaviors (as, for example, metformin, dietary restriction or antioxidants) can restore normality to mitochondrial and endothelial function, preserving the tissue's function and averting the aforementioned complications.

Keywords: type 2 diabetes mellitus, oxidative stress, mitochondrial dysfunction, inflammation, diabetic vascular complications, therapeutics

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the 21st century's global public health problems, with estimates of the affected population reaching 425 million people (IDF, 2017). The global prevalence of diabetes is rapidly growing in such a way that, according to the International Diabetes Federation (IDF) estimates, by 2045 a total of 629 million people will have diabetes (IDF, 2017). The increase

in diabetes prevalence is driven in large part by increasing rates of obesity and aging of the global population as well as changes in lifestyle related to unhealthy eating habits and sedentarism, with significant costs to healthcare systems. In 2017, diabetes caused more than 477 thousand deaths among adults in Europe alone; 32.9% of these deaths were of people under the age of 60 (IDF, 2017). In Portugal, according to data from the Annual Report of the Portuguese National Diabetes Observatory, the estimated prevalence of diabetes in 2015 was 13.3% (out of a population of 7.7 million individuals aged 20–79 years), meaning that more than 1 million Portuguese citizens in this age group have diabetes, of which 5.8% remain undiagnosed (Sociedade Portuguesa de Diabetologia, 2016). Considering the constantly increasing socio-economic and public health impact of diabetes and its complications, it is therefore critical to identify the underlying causes of diabetes to discover further measures of effective prevention and treatment (Tol et al., 2013).

Type 2 diabetes mellitus is a complex metabolic disorder associated with hyperglycemia, caused by defects in insulin secretion and/or action (Bergman, 2013; Alam et al., 2014). Over time, hyperglycemia induces toxic effects in virtually all of the organs of the body, of which the vascular system is particularly affected, resulting in multiple complications either at the microvascular level (retinopathy, nephropathy and neuropathy) or at the macrovascular level (stroke, coronary heart disease, acute myocardial infarction and peripheral vascular disease) (Calcutt et al., 2009; Gray and Jandeleit-Dahm, 2014; Heinonen et al., 2015). Approximately 40% of people with diabetes have late complications resulting from their disease progressing silently before the diagnosis is performed or even completed (IDF, 2015).

The pathophysiology of the link between T2DM and vascular complications is complex and multi-factorial. In fact, the precise mechanism by which T2DM leads to the development of these complications is complex and not yet fully elucidated, but seems to be strongly related with the toxic effects derived from hyperglycemia as well as by hyperlipidemia originated from obesity – gluco and lipo toxicity, respectively. Hyperglycemia induces oxidative stress, namely via mitochondrial dysfunction and enhanced reactive oxygen species (ROS) generation, while hyperlipidemia contributes to the release of pro-inflammatory cytokines by the adipocyte tissue. The consequent oxidative stress and low-grade-inflammation have been considered major contributors for the progression of T2DM and its complications (Santilli et al., 2015). In addition to the hyperglycemia and hyperlipidemia-induced toxicity, insulin resistance and hypertension promote damage at the level of blood vessel walls, which are manifested through the development of endothelial dysfunction (van den Oever et al., 2010), a condition that precedes the early development of micro and macrovascular diseases and complications (Cade, 2008).

Mitochondria play a key role in metabolic processes in all cells within an organism. Along with the famous ATP-generating oxidative phosphorylation, these organelles are critical for calcium chelation, biosynthetic pathways, ROS generation and cell death amongst many others. Unsurprisingly, interference and hampering of mitochondrial function is a hallmark of countless pathologic conditions and a central event of the progression of many diseases (Duchen, 2004). Depending on the endothelium location and functions, mitochondrial content of endothelial cells (EC) varies dramatically, as so does their function. Furthermore, their intracellular distribution is also reflective of their role within the cell (Park et al., 2011). As such, it is evident that mitochondrial dysfunction is at the core of endothelial injury, typically by inflammation, oxidative stress, cell death and loss of tissue function (Tang et al., 2014).

Even though there is a clear association between hyperglycemia and diabetic complications, the benefits of strict glycemic control on micro and macrovascular complications have been questioned, and interventions able to protect the organs targeted by diabetes are mandatory. Therapeutic strategies targeting oxidative stress, mitochondrial dysfunction and inflammation might be crucial to hinder the progression of vascular complications of diabetes. This review summarizes the contribution of the relationship between oxidative stress, mitochondrial oxidative damage and inflammatory aspects associated with endothelial dysfunction to the development of diabetes-related vascular complications. We also discuss therapeutic approaches that could prevent diabetic complications by specially targeting oxidative stress, mitochondrial impairment and inflammation pathways.

THE CROSSTALK BETWEEN OXIDATIVE STRESS AND INFLAMMATION IN THE PROGRESSION OF T2DM COMPLICATIONS

Diabetic patients frequently have several risk factors for development of vascular complications, including age, insulin resistance, dyslipidemia, hyperglycemia and hypertension. T2DM is not solely a metabolic disease but also a vascular one, characterized by chronic hyperglycemia and alterations of cellular homeostasis leading to vascular complications. The link between insulin resistance/hyperglycemia and endothelial dysfunction plays an important role in the development and progression of atherosclerotic disease in T2DM (Onat et al., 2006; Hadi and Suwaidi, 2007). Growing evidence suggests that hyperglycemia-induced oxidative stress promotes endothelial dysfunction by increased production of ROS, which plays a major role in the pathogenesis and progression of diabetic vascular complications (Pitocco et al., 2013; **Figure 1**).

Hyperglycemia, associated with insulin resistance and excessive free fatty acids (FFAs), initiates diabetic vascular complications through many metabolic and structural derangements, including in endothelial and vascular smooth muscle cells (VSMCs), compromising the vascular physiology and function. There are several proposed mechanisms underlying this hyperglycemia-evoked vascular damage, including increased production of advanced glycation end products (AGEs) and expression of their receptors RAGE (Receptor for AGEs), activation of protein kinase C (PKC) isoforms, as well as increased activation of the polyol and hexosamine fluxes, which induce increased mitochondrial ROS production,

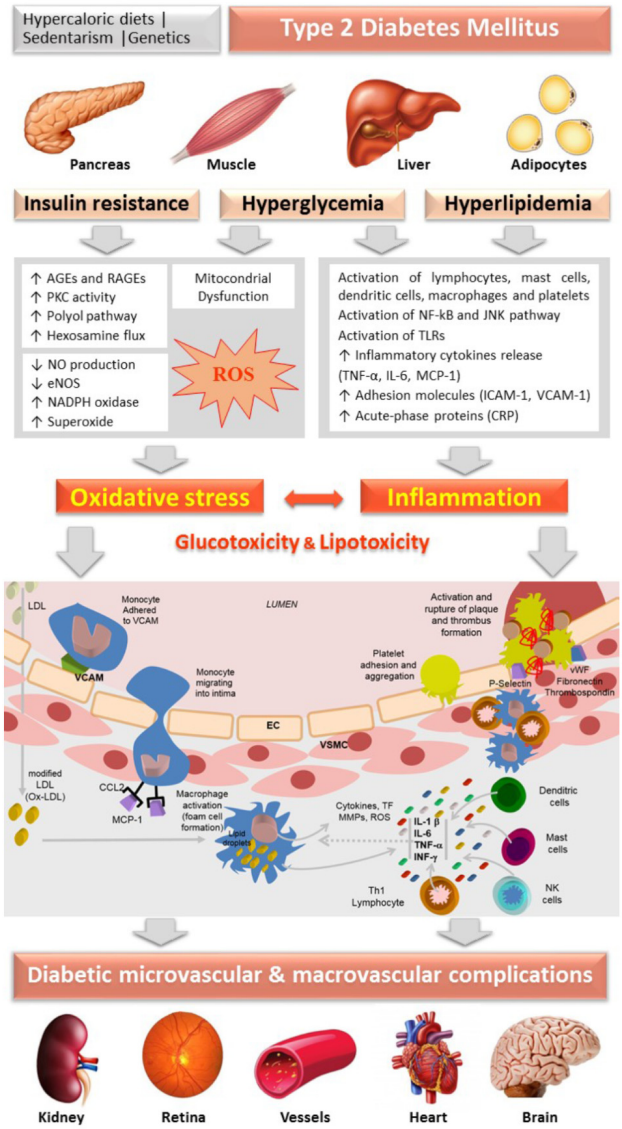

FIGURE 1 | Schematic diagram representing the central role of oxidative stress and inflammation, guided by insulin resistance, hyperglycemia and hyperlipidemia (gluco and lipo toxicity), in the vascular changes underlying the progression of micro and macrovascular complications of diabetes. AGEs, advanced glycation end products; CRP, C-reactive-protein; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; IFN, interferon; JNK, c-jun NH2-terminal kinase; LDL, Low Density Lipoprotein; MCP-1 (CCL-2), Monocyte chemotactic protein-1; NF-κB, nuclear factor-κB; NO, nitric oxide; PKC, protein kinase C; RAGEs, receptor for AGEs; ROS, reactive oxygen species; TLR, Toll-Like Receptor; TNF-α, tumor necrosis factor-alfa; VSMC, vascular smooth muscle cells; VCAM-1, vascular cell adhesion molecule; vWF, von Willebrand factor.

non-enzymatic glycation of proteins and auto-oxidation of glucose. The activation of these pathways resulting from prolonged hyperglycemia induces glucotoxicity (Brownlee, 2001; Rolo and Palmeira, 2006).

The activation of the polyol pathway contributes to oxidative stress as it causes nicotinamide adenine dinucleotide phosphate (NADPH) depletion at the consequent decrease in intracellular reduced glutathione levels (Pitocco et al., 2013). In an environment of hyperglycemia, aldose reductase reduces glucose to sorbitol, which is subsequently oxidized to fructose. In the process of reducing the high intracellular glucose content, aldose reductase consumes NADPH, which is the cell's main reducing agent and is also essential for the regeneration of the intracellular antioxidant glutathione (Brownlee, 2001, 2005).

Advanced glycation end products are generated by non-enzymatic glycosylation of proteins or lipids after prolonged exposure to glucose (Tamura et al., 2003). The formation of AGEs contributes to oxidative stress in several cell types through the interaction with their receptor, mainly by activation of NADPH oxidase and by the activation of the nuclear transcription factor kappa-B (NF-kB) pathway, leading to inflammatory and thrombogenic alterations that contribute to the pathogenesis of vascular diabetic complications (Brownlee, 2005; Giacco and Brownlee, 2010; Yamagishi et al., 2012). These glycated proteins accumulate on the vessels' walls exposed to hyperglycemia, altering the structural integrity of the vascular wall and neutralizing nitric oxide (NO), substantially affecting endothelial function. The production of AGEs is also responsible for the decrease in endothelial NO synthase (eNOS) expression as well as macrophage-mediated inflammation in the vessels' walls. In addition, they increase endothelin-1 (ET-1), an endothelium-derived potent vasoconstrictor, in EC (Santilli et al., 2015). Furthermore, circulating AGEs appear to react directly with lipoproteins, especially low-density lipoproteins (LDL), inducing structural alterations and damaging the mechanisms of LDL-receptor-mediated particle removal at tissue level (Bucala et al., 1994). Indeed, these products have been identified in atherosclerotic plaques, suggesting a possible role of AGEs in the pathophysiological of cardiovascular complications in diabetic patients (**Figure 1**).

The activation of PKC contributes to the production of superoxide anion in vascular EC (Ceriello et al., 2004; Paneni et al., 2013). The hyperglycemic environment causes chronic elevation of diacylglycerol (DAG) levels in EC, with membrane translocation of PKC isoforms. PKC phosphorylates NADPH oxidase, stimulating the production of superoxide, further aggravating oxidative stress (Paneni et al., 2013). This activation interferes with the biosynthesis of NO by decreasing eNOS activity through increasing the phosphorylation, also leading to an increment of ET-1 production, promoting increased vasoconstriction and platelet aggregation. In addition, it stimulates the synthesis of the extracellular matrix and promotes an inflammatory response through the activation of cytokines and adhesion molecules (Giacco and Brownlee, 2010; Paneni et al., 2013; Domingueti et al., 2016; **Figure 1**).

Another pathway that involves ROS generation is the production of NO, which plays a central role in the modulation of endothelial function, regulating vascular homeostasis and the innate immune system. In addition, NO generation by macrophages is elevated in various inflammatory conditions, including atherosclerosis (Bashan et al., 2009; Pitocco et al., 2013; Lundberg et al., 2015). The vasoprotective beneficial actions of NO include vasodilatation, increase in blood flow,

hypotension, inhibition of platelet aggregation and adhesion, as well as reduction of smooth muscle proliferation (Toda et al., 2010). Endothelial dysfunction refers to an imbalance in the release of NO or other vasodilatory factors and vasoconstrictor substances, and is related to the pathology of diabetes and related complications (Ouviña et al., 2001). Under diabetic conditions, endothelial dysfunction leads to impaired NO availability, namely by eNOS uncoupling, leading to its deficiency and increase in vascular resistance, contributing to atherogenesis (Johnson, 2012). The reduction in vascular NO bioavailability is related to its inactivation by ROS, in such a way that has been used as a biomarker of oxidative stress (Pitocco et al., 2010).

In T2DM, the main sources of oxidative stress are the mitochondria (Rolo and Palmeira, 2006; Asmat et al., 2016) (details are discussed in the next section). Briefly, the increase in the generation of mitochondrial ROS has been implicated as a mediator between hyperglycemia and its pathological consequences in the vessels, kidneys, neurons and retina (Bashan et al., 2009). Glucose can directly stimulate the overproduction of ROS, which leads to the activation of several enzymatic cascades resulting in mitochondrial dysfunction, including activation of NADPH oxidase, decoupling of NO synthases and stimulation of xanthine oxidase (Giacco and Brownlee, 2010; Pitocco et al., 2013).

Insulin resistance is also associated with endothelial dysfunction, resulting in a reduction of biosynthesis and biological activity of NO (Tessari et al., 2010). Endothelial dysfunction caused by insulin resistance may also be provoked by the activation of the extracellular-signal-regulated kinase/mitogenic protein kinase (MAPK) pathways, which is responsible for insulin signaling. Insulin is also responsible for modulating the activity of eNOS through the phosphatidylinositol-3-kinase/serine-threonine kinase (PI3K/AKT) pathway (Sena et al., 2013). Thus, it has been demonstrated that subjects that present a reduction in eNOS expression are more susceptible to developing insulin resistance (Capellini et al., 2010).

In recent years, several reports have indicated that oxidative stress plays a crucial role in the development of inflammatory state. These pathological conditions reinforce each other, establishing a vicious cycle capable of extending and propagating the inflammatory response, contributing to the pathogenesis of T2DM and several other diseases (Lugrin et al., 2014). Moreover, mediators of inflammation have been associated with T2DM progression, and patients newly diagnosed with this condition have high levels of acute-phase proteins and proinflammatory cytokines, when compared to non-diabetic subjects (Yu et al., 2011; Nunes et al., 2012; Akash et al., 2013). Furthermore, chronic low-grade inflammation associated with states of obesity plays an important role in the development of chronic complications of diabetes (Nunes et al., 2012).

Gluco and lipo toxicity induce inflammation by several mechanisms, including activation of NF-kB, which leads to recruitment and activation of immune cells (Aminzadeh et al., 2013). ROS-induced expression of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), results in inflammatory cells' recruitment (Kaneto et al., 2010). ICAM-1 is expressed in both the vascular endothelium and monocytes and, therefore, is considered a biomarker of both endothelial dysfunction and low-grade inflammation (Hubbard and Rothlein, 2000). Changes in NO, cytokines, acute-phase reactants and cellular adhesion molecules induced by the overproduction of ROS precede atherosclerosis (**Figure 1**).

Increased inflammation promotes increased migration of neutrophils and monocytes, causing increased production and release of cytokines and mediators of inflammation, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) as well as monocyte chemotactic protein 1 (MCP-1), among other pro-inflammatory proteins (Pedicino et al., 2013). C-reactive protein (CRP) is considered an acute phase inflammatory protein, synthesized by hepatocytes and predominantly regulated by IL-6 and TNF-α, thus being considered a sensitive and reliable marker of inflammatory state. Increased serum levels of CRP are also present in chronic inflammatory conditions such as atherosclerosis. Their levels are approximately tripled in the presence of risk of peripheral vascular diseases. CRP promotes the expression of adhesion molecules (ICAM-1 and VCAM-1), which facilitate the adhesion of monocytes and T cells to the arterial wall in the first steps of the atherogenic process. The high plasma concentrations of CRP increase the risk of cardiovascular events (peripheral vascular disease, myocardial infarction, stroke and death), even among adults who did not present previous chronic processes (Willerson and Ridker, 2004).

Increased levels of pro-inflammatory cytokines, including TNF-α, IL-6 and CRP, also contribute to the reduction of endothelial relaxation factor NO and to increased activity of ET-1, which causes vasoconstriction by targeting VSMCs. Thus, low-grade inflammation has been linked to increased vascular permeability, altered vasoregulatory responses and the adhesion of monocytes, neutrophils, and macrophages, resulting in cell damage. In addition, cytokine release stimulates the expression of plasminogen activator inhibitor type-1 (PAI-1), a pro-thrombotic protein associated with vascular homeostasis (van den Oever et al., 2010; van Hinsbergh, 2012). Elevated levels of PAI-1 may also impair fibrinolysis in patients with T2DM (Pandolfi et al., 2001).

The excess of FFAs influences the development of insulin resistance through the activation of Toll-Like Receptors 4 (TLR-4) on the plasma membrane, subsequently activating the inflammatory proteins c-Jun N-terminal kinase (JNK), IκB kinase and NF-κB. TNF-α, an adipokine secreted by the adipose tissue, is also capable of activating these inflammatory proteins (Stanley et al., 2011; Stagakis et al., 2012). The inflammatory response induced by these molecules implies the inhibition of eNOS by the reduced expression and kinase activity of the insulin receptor, leading to altered phosphorylation on tyrosine substrates (IRS-1), with a subsequent decrease of the PI3K pathway. This results in reduced NO production by EC; in addition, inhibition of glucose transporter type 4 (GLUT4) translocation to the plasma membrane causes a reduction of glucose uptake in peripheral tissues, thus promoting endothelial dysfunction and peripheral insulin resistance (de Alvaro et al., 2004; Fernández-Veledo et al., 2009).

Furthermore, in response to endothelial injury and inflammation, oxidized lipids from LDL particles (Ox-LDL) accumulate in the endothelial wall of arteries (Dokken, 2008). Monocytes then infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophages and the attraction of T-lymphocytes which, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation (Fowler, 2008). Overall, the cellular damage caused by oxidative stress is a trigger for inflammatory stress, which reciprocally stimulates the production of free radicals, closing the pathological cycle underlying progression of diabetes and of its serious vascular complications (Lamb and Goldstein, 2008; **Figure 1**).

MITOCHONDRIA-INDUCED ENDOTHELIAL CELL DYSFUNCTION IN DIABETES PROGRESSION

Endothelial cell mitochondria are highly dynamic organelles in location, number and activity. Typically, angiogenesis leads to a paradoxical increase in both glycolysis and fatty acid oxidation, since glucose is more readily available than oxygen but if fatty acid oxidation is impaired, so is vessel growth (De Bock et al., 2013; Schoors et al., 2015). However, there are no doubts about the effects of hyperglycemia as present in diabetes. Endothelial cells are extremely sensitive to high glucose levels, which leads to apoptosis and loss of tissue function (Triggle et al., 2012) and is associated with elevated mitochondrial fragmentation, altered mitochondrial ultrastructure and increased ROS generation (Pangare and Makino, 2012; Mishiro et al., 2014; **Figure 2**). Similar results were found in isolated mitochondria from diabetic mouse coronary EC. In fact, accompanying increased mitochondrial fission was an elevated quantity of mitochondrial fission-regulating proteins, such as DRP1 (Makino et al., 2010) and FIS1, the latter in diabetic humans (Shenouda et al., 2011), although this does not appear to be an ubiquitous process (Zhong and Kowluru, 2011). Interestingly, the expression of the mitochondrial biogenic program regulator PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) affects the response of EC mitochondria opposite fashions, depending on the source (Sawada et al., 2014; Craige et al., 2016). We speculate that this might be related to altered mitophagic processes, where the removal of damaged mitochondria is not viable to the cell. Clarification of mitochondrial dynamics and biogenesis in EC diabetic stress will certainly contribute to novel therapeutic approaches. Giving further relevance to the role of mitochondrial function in EC are the numerous studies highlighting how hyperglycemia leads to calcium overload and elevated pro-apoptotic protein BAX content, which result in higher levels of mitochondrial permeability transition induction, ending in apoptosis (Kowluru and Abbas, 2003; Detaille et al., 2005).

Diabetes is typically associated with obesity, where a majority of patients present high levels of LDL-cholesterol which, in its oxidized form, has been shown to contribute to mitochondrial dysfunction and EC apoptotic death (Vindis et al., 2005). Abnormally high generation of mitochondrial ROS in response to increased nutrient availability (as occurs in obesity and diabetes) also has a primordial source, the mitochondrial respiratory chain and, in particular, complexes I and III of the aforementioned chain. Excess nutrients are readily metabolized and generate reducing equivalents for membrane potential generation in the mitochondrial inner membrane (which is primarily used for driving ATP synthesis at Complex V or ATP synthase) (Rolo and Palmeira, 2006). However, the excessive reducing power far outreaches the cell's ATP needs, as membrane potential ($\Delta\Psi$) builds up (Teodoro et al., 2013). As a result, the redox reactions of the mitochondrial respiratory chain decelerate immensely, which results in more reduced respiratory complexes that have no problem in being oxidized by molecular oxygen, leading to heightened ROS generation. As such, a controlled leak of protons back to the mitochondrial matrix presents itself as an attractive target for dealing with excess nutrients. In fact, nature has already developed such a system in the form of uncoupling proteins (UCP). While UCP1 is typically associated (and present) in brown adipose tissue, where it is responsible for this tissue's famous role in thermogenesis (by dissipation of $\Delta\Psi$), other forms of U exist in many other tissues (UCP2 and 3 have been shown to naturally occur in EC), where their role appears to hinge on ROS management and prevention, as one of their more efficient activators are free fatty acids products from triglyceride breakdown (Davis et al., 2008). It has already been demonstrated that UCP2 overexpression in EC reduces fatty acid-caused ROS generation, inflammation and apoptosis (Lee et al., 2005; Koziel et al., 2012), which directly correlates with vasoconstriction, atherosclerotic plaque deposition and ischemic stroke (Szewczyk et al., 2015). Further strengthening these observations is the report that increased ROS generation leads to higher rates of UCP2 expression in EC, which is dependent on the activation of AMP-activated protein kinase (AMPK) and PGC-1α (Valle et al., 2005; Szewczyk et al., 2015). Also, by removing UCP2, ROS, and $\Delta\Psi$ are increased, which leads to an inflammatory activation and increased EC death (Koziel et al., 2015; Szewczyk et al., 2015). The mechanism by which UCP2 further alters EC response to stress appears to hinge on p53 and its regulation of mitochondria, which is activated by ROS when UCP2 is absent or inactive (Shimasaki et al., 2013). These experimental data seem to gain strength from human diabetic patients carrying a mutation that leads to elevated UCP2 expression, which protects against the increased coronary risk (Cheurfa et al., 2008). Further studies on the matter of UCP2 and EC in diabetes have been reviewed before (Szewczyk et al., 2015).

THERAPEUTIC STRATEGIES

As briefly reviewed, oxidative stress, inflammation and mitochondrial dysfunction are closely linked with endothelial dysfunction, which is critical in the progression of micro and macrovascular diabetic complications (**Figure 1**). Thus, therapeutic strategies targeting these biological mechanisms could be pivotal to manage diabetes and its serious complications.

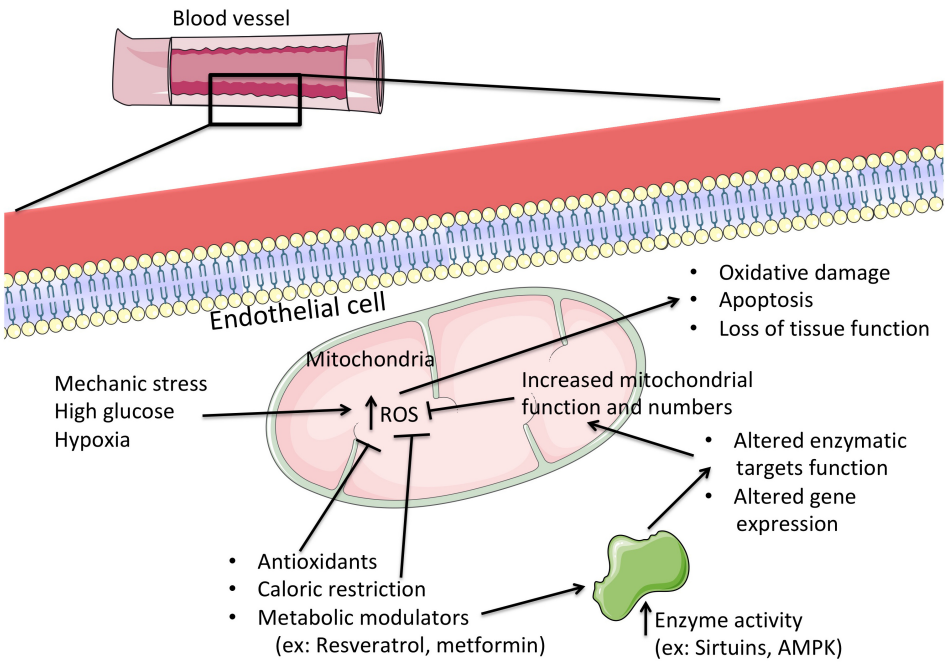

FIGURE 2 | The central role of mitochondria in the progression or prevention of hyperglycemic injury in endothelial cells. When challenged with abnormally high levels of glucose, endothelial cells progressively lose biochemical and functional capabilities, which are perfectly aligned with the loss of mitochondrial function. Here, the excess nutrients lead to a deceleration of metabolic reactions and electron transport in the respiratory chain, leading to a higher generation of reactive oxygen species (ROS). These in turn, when excessive, wreak havoc on cellular structures and biochemical processes, which might ultimately lead to cell death and loss of tissue function. By directly targeting mitochondrial function (for example, by consumption of metabolically active agents or by restriction to caloric intake) one can lead to improved mitochondrial activity by both post-transcriptional and gene expression alterations to mitochondrial function, with concomitant prevention of excessive oxidative stress and preservation of cellular integrity and function. ROS, reactive oxygen species; AMPK, AMP-activated protein kinase.

Herein, we review the main antioxidant and anti-inflammatory effects associated with some of the most efficient antidiabetic non-pharmacological and pharmacological interventions. A thorough revision of this matter is found below, and a table highlighting the main features of this section is also provided (**Table 1**).

Lifestyle Interventions

Non-pharmacological antidiabetic strategies encompass nutritional guidance and implementation of balanced and low-calorie diets as well as promotion of physical activity. These cornerstone lifestyle interventions could be pivotal not only to prevent diabetes' appearance and/or progression but also to ameliorate vascular complications, which coincide with a multiplicity of beneficial effects, including antioxidant and anti-inflammatory properties.

Physical Exercise (Training)

It has been evidenced that regular physical exercise (training) improves metabolic health due to improvement in glycemic control and insulin sensitivity, as well as due to a positive impact on abdominal circumference and visceral fat (de Lemos et al., 2007, 2009). Regular physical activity also favorably modulates other cardiovascular risk factors associated with T2DM, including blood pressure and lipid profile, demonstrated by the reduction of triglycerides, total-cholesterol and LDL-cholesterol levels together with the increase of high density lipoprotein (HDL)-cholesterol (Bassuk and Manson, 2005; de Lemos et al., 2007). In addition, physical exercise improves vascular function, namely by enhancing NO bioavailability (Green et al., 2004; Kwon et al., 2011). During physical exercise, an increased formation of free radicals is observed, mainly due to increased O_2 consumption by active tissues (Teixeira-Lemos et al., 2011; Vierck et al., 2012; Radak et al., 2013). Most of this O_2 consumed is used in the mitochondria for oxidative phosphorylation, where it is reduced to water; however, a small fraction (of about 2–5%) is converted into ROS (Teixeira-Lemos et al., 2011). Chronic physical activity of moderate intensity (training) positively alters the oxidative homeostasis of cells and tissues due to a reduction of basal levels of oxidative damage and to an increase in oxidative stress resistance (Cooper et al., 2002). In fact, regular exercise leads to an adaptation in antioxidant capacity, as viewed by the increment of superoxide dismutase and glutathione peroxidase activities, thus protecting cells against damage caused by oxidative stress (Teixeira-Lemos et al., 2011; Radak et al., 2013).

Furthermore, long-term training is an efficient anti-inflammatory measure, expressed by the reduction of pro-inflammatory mediators, such as CRP, interleukin-6 and TNF-α, and simultaneously by the increment of anti-inflammatory cytokines such as IL-4, IL-10 and adiponectin

TABLE 1 | Therapeutic strategies targeting some of the biological mechanisms (e.g., oxidative stress, inflammation and mitochondrial dysfunction) underlying endothelial dysfunction, which is critical in the progression of micro and macrovascular diabetic complications.

Therapeutic strategies	Main outcomes	References
Lifestyle interventions		
Physical exercise (training)	Improves glycemic control, insulin sensitivity, blood pressure, lipid profile, vascular function (NO availability); antioxidant and anti-inflammatory activity.	Green et al., 2004; Bassuk and Manson, 2005; de Lemos et al., 2007, 2009; Balducci et al., 2010; Kawanishi et al., 2010; Kwon et al., 2011; Teixeira-Lemos et al., 2011; Radak et al., 2013; Robinson et al., 2015.
Dietary interventions	Improves glycemic control, lipid profile and endothelial function; protects against atherosclerosis and reduces cardiovascular risk; antioxidant, anti-inflammatory and antifibrotic properties.	Gey, 1998; Barclay et al., 2007; Aguirre and May, 2008; Abbatecola et al., 2009; Nazıroğlu et al., 2010; Cho et al., 2013; Wang et al., 2013; Yan et al., 2013; Weickert and Pfeiffer, 2018.
Oral antidiabetics drugs		
Metformin	Hypoglycemic activity, improvement of insulin sensitivity and cardiovascular risk profile; amelioration of vascular dysfunction (e.g., by restoring NO availability and inhibiting AGEs formation); antioxidant and anti-inflammatory properties.	Ersoy et al., 2008; Yoshida et al., 2009; Sena et al., 2011; Kelly et al., 2012; Krysiak and Okopien, 2012, 2013; Batchuluun et al., 2014; Cheang et al., 2014; Vasamsetti et al., 2015; Triggle and Ding, 2017.
Thiazolidinediones	Hypoglycemic activity, improvement of insulin sensitivity and beneficial modulation of inflammatory, oxidative and endothelial vascular functions.	Cheng and Fantus, 2005; Sourij et al., 2006; Derosa and Sibilla, 2007; Dandona et al., 2008; Orasanu et al., 2008; Zhao et al., 2010; Hamblin et al., 2011; Jin et al., 2016.
Incretin-based therapies	Insulinotropic effects, gastric emptying delaying and reduction of endogenous glucose production by inhibiting glucagon secretion; reduction in blood pressure; improvement in endothelial dysfunction; extra-pancreatic cytoprotective properties, including anti-inflammatory, antioxidant and anti-apoptotic (e.g., against diabetic nephropathy and retinopathy.	Courrèges et al., 2008; Bergenstal et al., 2010; Ferreira et al., 2010; Hattori et al., 2010; Mega et al., 2011, 2014, 2017; Gonçalves et al., 2012, 2014; Lee et al., 2012; Shiraki et al., 2012; Dai et al., 2013; Ishikawa et al., 2014; Marques et al., 2014; Nakamura et al., 2014; Sun et al., 2015.
SGLT-2 inhibitors	Decrease blood glucose levels, body weight and blood pressure; cardiovascular and renal protection via anti-inflammatory and antioxidant effects; attenuate atherosclerotic lesion formation, with reduction of cardiovascular morbidity and mortality.	Hasan et al., 2014; Chilton et al., 2015; Ojima et al., 2015; Scheen, 2015; Tikkanen et al., 2015; Zinman et al., 2015; Leng et al., 2016.
Mitochondrial-targeting strategies		
Antioxidants/ ROS scavengers (e.g., MitoQ-TPP and TEMPOL)	Improve vascular prognosis in diabetics by contributing to reduce oxidative stress and blood pressure, thus improving vascular relaxation. Protect against hypertension by preserving EC function, which correlates with improved cardiac function; in addition, prevent inflammation at atherosclerotic plaque sites.	Graham et al., 2009; Dikalova et al., 2010; Mercer et al., 2012.
Caloric restriction	Increases mitochondrial biogenesis and efficiency, and reduces vascular inflammation thus improving EC dysfunction; ameliorates atherosclerosis, diminishes ROS generation, and overall reduces plaque deposition, hypertension and other cardiovascular complications in humans.	Guo et al., 2002; Fontana et al., 2004; Nisoli et al., 2004; López-Lluch et al., 2008; Lefevre et al., 2009; Finckenberg et al., 2012.
Metabolic modulators: (i) Sirtuin 1 activators (e.g., resveratrol) (ii) AMPK activators (e.g., metformin) (iii) PPARγ activators (e.g., pioglitazone)	(i) Decreases p66Shc overexpression induced by hyperglycemia; p66Shc promotes oxidation of several targets in the EC mitochondria, thus contributing to endothelial dysfunction. Resveratrol leads to AMPK activation, eNOS increment, reduction of ROS generation and plaque deposition, culminating in improved EC function. (ii) Inhibit the induction of the mitochondrial permeability transition leading to the prevention of EC apoptosis and endothelial loss of function. (iii) Activates PGC-1α, leading to improved mitochondrial biogenesis in EC, thus contributing to improve endothelial dysfunction.	Wang et al., 2005; Schulz et al., 2008; Csiszar et al., 2009; Fujisawa et al., 2009; Zhou et al., 2011; Price et al., 2012.

(Balducci et al., 2010; Teixeira-Lemos et al., 2011; Radak et al., 2013). One hypothesized mechanism through which training might exert an anti-inflammatory activity may be related to the modulation of TLR-dependent pathways (Kawanishi et al., 2010; Robinson et al., 2015) as viewed by the reduced expression of TLR2 and TLR4 in monocytes, lymphocyte and neutrophils in obese adults with a higher risk for developing T2DM after training of moderate intensity (Robinson et al., 2015).

Dietary Interventions

Lifestyle intervention programs in T2DM patients, especially in those who are obese, have been mainly focused on the

reduction of caloric intake and on body weight loss, which seem *per se* to have a major beneficial impact on glycemic control and cardiovascular risk (Buse et al., 2006). Several studies have been carried out to identify the perfect combination of macronutrients able to prevent the onset of CVD; nevertheless, the best combination of proteins, carbohydrates and lipids varies according to the individual, which makes it difficult to define a universal food plan for this type of patients (Franz et al., 2004; Grundy et al., 2005). However, it is known that some foods (and nutrients contained therein) exert anti-diabetic and vasoprotective properties, in particular because they have antioxidant and anti-inflammatory effects.

Numerous studies have shown that diets rich in whole grains, omega (ω3) fatty acids and fibers, associated with a low consumption of *trans* fatty acids carbohydrates and cholesterol, are recommended strategies to improve the lipid profile and to reduce cardiovascular risk in T2DM patients with a high glycemic index (Barclay et al., 2007; Abbatecola et al., 2009; Cho et al., 2013; Weickert and Pfeiffer, 2018). More precisely, there is a wide variety of antioxidative substances found in food, mainly in fruits and vegetables, which can synergistically act in the protection of cells and tissues (Blomhoff, 2005; Halvorsen et al., 2006). Several epidemiological studies have suggested a direct association between vitamin E intake and reduction of cardiovascular morbidity and mortality (Wang et al., 2013), although limited information is available regarding the impact of vitamin E supplementation on T2DM patients (Boshtam et al., 2005; Giannini et al., 2007). On the other hand, a combination of vitamins C and E seems to be an effective strategy due to inhibition of lipid peroxidation and protection against DNA damage, as previously reported (Gey, 1998; Nazıroğlu et al., 2010). Vitamin C can afford protection in several types of vascular cells involved in the process of atherosclerosis: ascorbate helps to prevent endothelial dysfunction, stimulates the synthesis of type IV collagen and increases proliferation, while also inhibiting differentiation and proliferation of vascular smooth muscle cells in areas of injury and reducing oxidative stress in macrophages (Aguirre and May, 2008). Antioxidants can inhibit lipid peroxidation directly by scavenging the peroxide radicals and indirectly by regenerating the active form of other antioxidant compounds, like vitamin E, flavonoids and glutathione (Aguirre and May, 2008).

Consumption of ω3-polyunsaturated fatty acids (PUFAs) seems to provide cardioprotection in diabetic conditions due to pleiotropic properties, including those of an antioxidant, anti-inflammatory and antifibrotic nature. Regarding the impact on inflammation, ω3-PUFAs were associated with attenuation of both TLR4 and TNF-α-mediated pro-inflammatory signaling in macrophages and inhibition of the inflammasome via effects on NLRP3 in high-fat diet (HFD)-induced diabetic mice (Yan et al., 2013).

Oral Antidiabetics Drugs

Metformin

Metformin is a first-line pharmacological treatment for most T2DM patients. This anti-hyperglycemic agent is an activator of AMPK and suppresses hepatic glucose synthesis and improves insulin sensitivity by enhancing insulin-stimulated peripheral glucose uptake (Yoshida et al., 2009). In addition to its hypoglycemic effect, other beneficial effects of this drug are being studied, including its role on the prevention of vascular complications. Clinical trials that have enrolled overweight adult patients with or without T2DM support the decrease of cardiovascular risk profile induced by metformin (De Jager et al., 2005; Ersoy et al., 2008; Kelly et al., 2012); however, the underlying mechanism of metformin's cardioprotective actions remains to be fully understood. Emerging evidence suggests that metformin boasts both direct and indirect antioxidant and anti-inflammatory properties (Sena et al., 2011; Krysiak and Okopien, 2012, 2013; Vasamsetti et al., 2015). The activation of AMPK pathways plays a pivotal role on its pharmacological effects and might explain the variety of pleiotropic actions of this drug. In fact, several mechanisms that explain metformin's beneficial actions have been proposed, including NF-kB inhibition, NO production incrementation and inhibition of AGEs formation.

The antioxidative effect of metformin may be related with the reduction of DAG levels, inhibition of PKC translocation to the cellular membrane, and suppression of the NADPH oxidase activity, leading to reduced ROS production (Piwkowska et al., 2010; Batchuluun et al., 2014). In obese mice fed with a HFD, treatment with metformin improved endothelial function by reducing endoplasmic reticulum stress, superoxide production and by increasing NO bioavailability (Cheang et al., 2014). Metformin has been shown to directly inhibit ROS production from complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial electron transport chain (Owen et al., 2000) and to increase the AMP/ATP ratio. Moreover, Sartoretto et al. (2005) reported that metformin increases NO activity, but not expression, and that it improves microvascular reactivity to histamine, bradykinin or acetylcholine of arterioles and venules in T2DM animal models. A number of basic and clinical studies investigated the effect of metformin on endothelium-dependent vascular function (Mather et al., 2001; Sena et al., 2011; Triggle and Ding, 2017). In particular, Sena et al. (2011) have reported that metformin restored endothelial function by enhancing NO bioavailability and reducing oxidative stress and inflammation in the aortic rings of normal and high fat-fed diabetic GK rats. The authors showed that metformin treatment reduced monocyte chemoattractant protein-1 (MCP-1/CCL2) levels that were increased in T2DM rats, indicating an inhibition of early inflammation in the aorta (Sena et al., 2011).

Previous studies have described that metformin inhibits proinflammatory responses in vascular endothelial and VSMCs (Hattori et al., 2006; Isoda et al., 2006). Hattori et al. (2006) have reported that metformin inhibited cytokine-induced NF-kB activation via AMPK activation in human vascular EC. Another report demonstrated that metformin may attenuate Ox-LDL-induced proinflammatory responses in monocytes and macrophages and inhibit monocyte-to-macrophage differentiation (Huangfu et al., 2018). Vasamsetti et al. (2015) reported that metformin inhibits monocyte-to-macrophage differentiation by decreasing STAT3 phosphorylation through increased AMPK activation and leads to a reduction of

proinflammatory cytokine production. Furthermore, clinical studies also suggest that metformin may modulate the inflammatory status as viewed by the reduction of several proinflammatory cytokines (Krysiak and Okopien, 2012, 2013). Krysiak and Okopien (2013) demonstrated that metformin could reduce the monocyte secretion of TNF-α, IL-1β, IL-6, MCP-1, and IL-8, as well as plasma CRP levels, in patients with impaired fasting glucose. Another study with obese diabetic patients showed that after 12 weeks of metformin therapy, there was a decrease in PAI-1 and in vascular endothelial growth factor (VEGF) (Ersoy et al., 2008). Moreover, De Jager et al. (2005) also observed, in patients with T2DM, that treatment with metformin for 16 weeks reduced levels of plasma VCAM-1, soluble E-selectin, PAI-1, and von Willebrand factor (vWF), whereas markers of inflammation were unaffected. Thus, in clinical trials the anti-inflammatory effects of this drug remain to be fully clarified.

Thiazolidinediones

Thiazolidinediones (TZDs), including rosiglitazone and pioglitazone, are a class of drugs known to improve insulin sensitivity in peripheral tissues. These drugs bind to and activate the peroxisome proliferator-activated receptor gamma (PPARγ), which is found in the liver, muscle, heart, kidney, and adipose tissue and is involved in the regulation of expression of insulin-sensitive genes, which are crucial to glucose and lipid metabolism. Apart from the hypoglycemic effects, TZDs have showed an ability to modulate inflammatory, oxidative and vascular functions.

The anti-inflammatory action of TZDs has been suggested by the increment of adiponectin and parallel reduction of cytokine production from the adipose tissue, such as TNF-α and resistin (Cheng and Fantus, 2005). Furthermore, it was suggested that pioglitazone decreases inflammation partly through inhibiting AGE-induced classical macrophage polarization in diabetic HFD fed mice (Jin et al., 2016). A meta-analysis showed that pioglitazone and rosiglitazone significantly decreased serum CRP levels in subjects with and without diabetes, irrespective of the effects on glycemia (Zhao et al., 2010). Previous studies have suggested an improved endothelial function with TZD treatment (Derosa and Sibilla, 2007; Dandona et al., 2008). In *in vitro* and *in vivo* studies, pioglitazone protects against oxidative stress, reduces blood pressure and decreases VCAM-1 expression on EC through modulation of NF-κB activity via a PPARα-dependent mechanism (Orasanu et al., 2008; Hamblin et al., 2011). Concurrent with this, pioglitazone was also shown to improve endothelial function in non-diabetic individuals with coronary artery disease, suggesting that pioglitazone exerts a direct effect on the endothelium (Sourij et al., 2006). In addition, in obese and diabetic patients, pioglitazone has been shown to reduce arterial stiffness and reduce blood pressure and CRP levels, independently of changes in glycemic control (Satoh et al., 2003; Clarke et al., 2017). Although there are several studies describing the beneficial vascular effects of PPAR agonists, including the PROACTIVE (Prospective Pioglitazone Clinical Trial in Macrovascular Events) trial, the cardiovascular effects of TZDs are still not well understood, and current clinical evidence leaves this hypothesis unproven. The recognition of an increased risk of myocardial infarction and heart failure in some patients using rosiglitazone therapy (Nissen and Wolski, 2007), which caused its withdrawal in Europe and limited use in the United States, added controversy to this subject, leading to a need for further clarification.

Incretin-Based Therapies

Glucagon-like peptide-1 (GLP-1) is an incretin hormone primarily synthesized by the endocrine L cells of the gastrointestinal tract during and after food intake and stimulates glucose-dependent insulin secretion by pancreatic β cells. The insulin secretory response of incretins is known as the incretin effect, and accounts for at least 50% of postprandial insulin secretion. In addition to its insulinotropic effects, GLP-1 delays gastric emptying and also reduces endogenous glucose production by inhibiting glucagon secretion by pancreatic α-cells (Triplitt et al., 2006). This incretin is rapidly metabolized by the ubiquitous enzyme dipeptidyl peptidase-IV (DPP-IV) to inactive metabolites, which are then eliminated by the urine (Ranganath, 2008), resulting in the inactivation of the incretin effect (Holst, 2007). The recognition, and further characterization of an impaired incretin effect (known as the incretin defect) in diabetic patients was the base for the development of a new class of antidiabetic agents, the incretin-based therapies, which includes DPP-IV inhibitors and GLP-1 receptor (GLP-1R) agonists (Gallwitz, 2005). Over the last years, extra-pancreatic protective effects, behind glucose-insulin control, have been suggested in distinct vascular conditions (Scirica et al., 2013; Godinho et al., 2015).

In addition to regulating glucose and metabolic control, GLP-1 has a potential beneficial effect on multiple pathways involved in atherogenesis. Although the mechanisms of vascular effect are still unclear, it seems that the protective action of GLP-1 may be related to an improvement in endothelial dysfunction through its anti-inflammatory and antioxidant effects (Nyström et al., 2004). Several studies performed in T2DM patients showed a positive impact of GLP-1R agonist treatment in endothelial function (Nikolaidis et al., 2004; Sokos et al., 2006). Liraglutide and exenatide therapy were able to reduce the levels of PAI-1 inhibitor, a regulator of plasminogen activation implicated in EC dysfunction (Courrèges et al., 2008; Liu et al., 2009; Bergenstal et al., 2010). Although the exact mechanisms remain to be fully elucidated, several studies reporting on treatment with these two GLP-1R agonists have consistently demonstrated a reduction in blood pressure in patients with T2DM as well as a reduction in brain natriuretic peptide (BNP) levels (Courrèges et al., 2008; Bergenstal et al., 2010; Sun et al., 2015). Moreover, in a human vascular endothelial cell line, liraglutide inhibited TNF-α, fibrinolysis inhibitor PAI-1, and the mRNA and protein levels of VCAM-1 and ICAM-1 (Liu et al., 2009). Likewise, it has been reported that liraglutide exerts marked antioxidant and anti-inflammatory effects on vascular EC by increasing NO production, with inhibition of PKC-α, NADPH oxidase, NF-κB and JNK signaling, while also leading to the overexpression of superoxide dismutase (SOD) and catalase protective antioxidant enzymes (Hattori et al., 2010; Shiraki et al., 2012). Additionally,

also in *in vitro* studies, lipopolysaccharide (LPS)-stimulated inflammatory responses were inhibited by exendin-4, a GLP-1 analog, in cardiomyoblasts (Chen et al., 2012) and in human peripheral mononuclear cells (PBMCs) (Hogan et al., 2014). In ob/ob mice, treatment with recombinant adenovirus producing GLP-1 inhibited macrophage infiltration and adipose tissue expression and production of IL-6, TNF-α, and MCP-1 (Lee et al., 2012). Dai et al. (2013) reported that liraglutide protects against atherogenesis by the reduction of Ox-LDL-induced mitochondrial ROS in human aortic VSMCs. In addition, these inhibitory effects were abrogated by the overexpression of lectin-like oxidized low-density lipoprotein scavenger receptor-1 (LOX-1), suggesting that LOX-1 plays an important role in the antioxidant effects of GLP-1R agonists.

Dipeptidyl peptidase-IV inhibitors, another group of incretin-based therapies, increase endogenous incretin levels availability (such as GLP-1) and exert several insulinotropic effects that contribute to maintain normal glucose levels (Godinho et al., 2015). In particular, DPP-IV inhibitors are able to enhance glucose-stimulated insulin secretion, inhibit glucagon secretion and promote beta-cell proliferation and survival (Brubaker and Drucker, 2004). Apart from the insulin-dependent effects, DPP-IV inhibitors, namely sitagliptin (the first drug of this new class of antidiabetic agents), exert extra-pancreatic cytoprotective properties. Our group has already demonstrated antioxidant and anti-inflammatory effects of sitagliptin in animal models of diabetic nephropathy and diabetic retinopathy (Ferreira et al., 2010; Mega et al., 2011, 2014, 2017; Gonçalves et al., 2012, 2014; Marques et al., 2014). In models of type 1 and type 2 diabetes, sitagliptin was able to ameliorate diabetic retinopathy by preventing nitrosative stress, inflammation and apoptosis in retinal cells and by exerting beneficial effects on the blood retinal barrier (Gonçalves et al., 2012). In the Zucker Diabetic Fatty (ZDF) rat, a model of obese T2DM, sitagliptin ameliorated diabetic nephropathy, which was accompanied by an antioxidant effect and by a significant reduction in inflammatory state and cell death by apoptosis (Mega et al., 2011, 2017; Marques et al., 2014).

Other studies in both animals and humans have suggested similar protective properties in other tissues and conditions, including a positive impact on vascular endothelium with anti-atherosclerotic action (Ishikawa et al., 2014; Nakamura et al., 2014), usually based on the anti-inflammatory activity of these antidiabetic agents (Lee et al., 2012; Satoh-Asahara et al., 2013). In fact, T2DM patients treated during 12 weeks with sitagliptin presented a reduction in circulating inflammatory markers, like CRP and IL-6 and reduced monocyte expression of mRNA transcripts associated with inflammation (Makdissi et al., 2012). Furthermore, treatment with sitagliptin improved the inflammatory state, vascular endothelial function and prevented the progression of carotid atherosclerosis in a dose-dependent manner independent of its glucose-lowering effects (Ayaori et al., 2013). Considering the direct effects regarding the glycemic control and the pleiotropic effects on extra-pancreatic tissues, DPP-IV inhibitors as well as GLP-1R agonists are promising options for managing diabetes and vascular complications.

SGLT-2 Inhibitors

Selective sodium/glucose co-transporter 2 (SGLT-2), is a high-capacity, low-affinity glucose transport protein, which is primarily found in the kidney but also in the intestine (Hasan et al., 2014). This glucose transporter is responsible for about 90% of glucose reabsorption in the kidney (Vallon, 2011). Empagliflozin, canagliflozin, and dapagliflozin are SGLT-2 inhibitors, also known as gliflozins, currently available in Europe and in the United States for diabetes management. In diabetic conditions, the kidneys increase expression of SGLT-2 and, unlike the liver, increased glucose ingestion elevates kidney gluconeogenesis even in diabetic patients (Meyer et al., 1998; Hasan et al., 2014). SGLT-2 inhibitors exert their effect on the kidneys, preventing reabsorption of glucose from the proximal tubules via SGLT-2 (Scheen, 2015). Clinical trials of SGLT-2 inhibitors have shown that these drugs decreased glucose levels independent of insulin (Scheen, 2015). SGLT-2 inhibitors may also have additional benefits related with weight loss and with reductions in blood pressure that occur because of the osmotic effect of glucose excretion and subsequent inhibition of the renin-angiotensin system (Scheen, 2015). Recently, a few studies indicated that SGLT-2 inhibitors may exert their cardiovascular and renal protection via anti-inflammatory and antioxidative effects (Ojima et al., 2015). Leng et al. (2016) found that dapagliflozin can attenuate the formation of atherosclerotic lesions, increase the stability of lesions, reduce the production of IL-1β by macrophage infiltration, and decrease mitochondrial ROS generation. These effects may be associated with an inhibitory effect on the NLRP3 inflammasome in diabetic atherosclerosis, which provides further evidence for its benefits in diabetic patients. Moreover, also in diabetic rats, empagliflozin was shown to improve hyperglycemia, reduce urinary excretion levels of tubular injury markers, decrease expression levels of oxidative stress biomarkers (AGEs and RAGE), and reduce inflammatory and fibrotic markers in the kidney, including MCP-1, ICAM-1, PAI-1, TGF-β (Ojima et al., 2015). The authors suggest that a blockade of SGLT-2 by empagliflozin might protect proximal tubular cells from glucotoxicity in diabetic nephropathy partly via suppression of the AGE-RAGE-mediated oxidative stress generation. Furthermore, empagliflozin given to patients with T2DM was shown to reduce both blood pressure and arterial stiffness (Chilton et al., 2015; Tikkanen et al., 2015), and data from the EMPA-REG OUTCOME (Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients–Removing Excess Glucose) trial showed that empagliflozin is the first antidiabetic drug compound to conclusively reduce cardiovascular morbidity and mortality (Zinman et al., 2015).

Mitochondrial-Targeting Strategies

By targeting mitochondrial ROS generation, it is possible to increase endothelial activity and improve endothelial dysfunction in diabetes (**Figure 2**). For example, it has been shown that the nicotinamide adenine dinucleotide phosphate oxidase

4 (NOX4) enzyme regulates mitochondrial ROS generation, leading to vessel relaxation and lower blood pressure (Santillo et al., 2015). This is further confirmed by the fact that higher levels of low-density lipoproteins, typically associated with obesity and diabetes, increase NOX activity, which leads to increased oxidative stress and cell death (Li et al., 2016). Also, NOX activity has been implicated in altered angiogenesis and increased susceptibility to hypoxia and stroke (Amanso and Griendling, 2012; Caja and Enríquez, 2017). Interestingly, NOX4 exists in EC and, in certain conditions, is present within mitochondria, where it can block the respiratory chain complex I, leading to increased ROS generation (Kozieł et al., 2013) and elevating the interest in this enzyme for diabetic-associated ROS management (Touyz and Montezano, 2012). Another regulator of mitochondrial ROS, with clear implications for endothelial dysfunction, is p66Shc, which responds to high glucose by migrating to mitochondria and inducing oxidation of key effectors, leading to cell death and loss of tissue function (Camici et al., 2007; Paneni et al., 2012). In particular, p66Shc can oxidize reduced cytochrome *c*, which accumulates in anoxic conditions, a prevalent situation in diabetes in certain tissues where cell enlargement (ex: adipocytes) is present (Giorgio et al., 2005). The NAD^+-dependent deacetylase Sirtuin 1 is a known regulator of mitochondrial function and cellular homeostasis (Price et al., 2012). It has already been demonstrated that EC-specific Sirtuin 1 activation decreases p66Shc overexpression by hyperglycemia, which is predictably related to improved endothelial function (Zhou et al., 2011).

Another factor of ROS-mediated EC injury is the oxidation of NO by ROS. Not only is NO removed (leading to increased vasoconstriction and thus hypoxia and further ROS generation, as well as mechanic injury to the vessel due to elevated blood pressure) but NO synthase activity is also compromised, resulting in even further ROS generation (Forstermann and Münzel, 2006). As such, antioxidant therapy or elevation of the natural antioxidant defenses in mitochondria has been shown to significantly improve vascular diabetic prognosis by contributing to reduced oxidative stress, vascular relaxation and reduced blood pressure (Dikalova et al., 2010).

However, since ROS has transitioned from simply toxic agents toward a signaling function, a careful approach to indiscriminate antioxidant therapy must be taken. In fact, mitochondrial ROS are implicated in normal physiological roles of EC, such as shear-stress-induced vasodilation, hypoxia handling, autophagy, and inflammation (Caja and Enríquez, 2017). Nevertheless, pathological surges in ROS generation are undoubtedly connected to loss of vascular function (Brownlee, 2001; Yu et al., 2006). This has been demonstrated by the use of mitochondrial-targeting ROS scavengers. Mitoquinone (mitoQ) attached to triphenylphosphonium (TPP) protected against hypertension by preserving EC function, which correlates with improved cardiac function (Graham et al., 2009), while it can also prevent inflammation at atherosclerotic plaque sites (Mercer et al., 2012). Similarly, the use of mitochondria-targeting TEMPOL, a known ROS scavenger, yielded similar results by decreasing hypertension (Dikalova et al., 2010).

A different approach hinges on preventing heightened ROS generation by limiting nutrient availability, whether by caloric restriction or agents that mime its effects. Unsurprisingly, calorie restriction increases mitochondrial biogenesis and efficiency (Nisoli et al., 2004; López-Lluch et al., 2008), which can be harnessed for management of diabetic-induced EC dysfunction. In fact, this is far from a novel idea, since Young and collaborators have shown that calorie restriction improves blood pressure in hypertense rats (Young et al., 1978), but it was recently confirmed that it reduces cardiac hypertrophy and vascular inflammation, in part by targeting mitochondrial function (Finckenberg et al., 2012). This is further demonstrated by the fact that caloric intake reduction improves atherosclerosis, diminishes ROS generation (Guo et al., 2002), and overall reduces plaque deposition, hypertension and other cardiovascular complications in humans (Fontana et al., 2004; Lefevre et al., 2009). These effects, unsurprisingly, involve the modulation of the activity of many previously discussed agents, such as Sirtuin 1 and AMPK, so much so that their activity modulating compounds are widely considered calorie restriction mimetics (Fontana et al., 2010). For example, the widely consensual Sirtuin 1 activator resveratrol (Price et al., 2012) leads not only to AMPK activation, as the activity of this protein elevates eNOS, but also reduces ROS generation and plaque deposition, culminating in improved EC function (Wang et al., 2005; Csiszar et al., 2009). Similarly, the known AMPK activator metformin inhibits the induction of the mitochondrial permeability transition, leading to the prevention of EC apoptosis and endothelial loss of function (Schulz et al., 2008). Finally, by activating PPARγ, the TZD pioglitazone activates PGC-1α, leading to improved mitochondrial biogenesis in EC. PGC-1α can also be activated by both AMPK and Sirtuin 1, which highlights the interaction and interconnection of these metabolic agents and pathways (Fujisawa et al., 2009).

The most common class of ion-selective channels are potassium transmembrane channels (Szewczyk et al., 2015). While the majority of these channels are present in the plasma membrane, they are also present in the inner mitochondrial membrane, where they regulate potassium fluxes and thus modulate various mitochondrial functions and parameters, such as membrane potential, ATP and ROS generation, mitochondrial volume and calcium import (Bernardi, 1999; Szabo and Zoratti, 2014). Given the accumulation of negative charges in the mitochondrial matrix, the positively charged potassium ions enter the mitochondria in favor of the gradient and cause a dissipation of membrane potential and thus decrease ROS generation. As expected, EC mitochondria have potassium channels where their activation decreases $\Delta\Psi$ and thus lead to vasodilation (Katakam et al., 2013), an effect that might be described by the apparent physical coupling of potassium channels with the respiratory chain (Bednarczyk et al., 2013).

CONCLUSION AND PERSPECTIVES

Vascular complications represent the major cause of morbidity and mortality in T2DM patients and are responsible for the lower

life expectancy of these subjects. Given the complexity of the mechanisms involved in disease appearance and progression, it is unlikely that a single therapeutic measure could efficiently control all the factors underlying the slow but consistent deregulation of micro and macro vascular beds. While some patients may greatly benefit from interventions based on changes in lifestyle habits, for the majority of T2DM subjects, pharmacological interventions are unavoidable and crucial. However, apart from the most recent trial with the SGLT-2 inhibitor empagliflozin (EMPA-REG OUTCOME), the extensive list of oral antidiabetic drugs already available for treating T2DM patients has failed to show consistent reduction in cardiovascular mortality, despite collectively, in mono and/or combined therapy, being able to provide good glycemic control. This evidence suggests that we still need to improve the knowledge about disease and complications in order to be able to develop newer targets of intervention. In order to circumvent the current limitations and improve our capacity to fight T2DM and its serious complications, there is growing interest in looking for some of the mechanisms that play a major role, such as hyperglycemia and hyperlipidemia-evoked oxidative stress, mitochondrial dysfunction and inflammation. By better controlling the causes of vascular disease(s) and targeting the mechanisms involved, with older or newer agents, we can expect improvements in the cardiovascular morbidity and mortality outcomes of T2DM patients.

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Age-Dependent Impairment of Neurovascular and Neurometabolic Coupling in the Hippocampus

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Neurovascular and neurometabolic coupling are critical and complex processes underlying brain function. Perturbations in the regulation of these processes are, likely, early dysfunctional alterations in pathological brain aging and age-related neurodegeneration. Evidences support the role of nitric oxide (•NO) as a key messenger both in neurovascular coupling, by signaling from neurons to blood vessels, and in neurometabolic coupling, by modulating O_2 utilization by mitochondria. In the present study, we investigated the functionality of neurovascular and neurometabolic coupling in connection to •NO signaling and in association to cognitive performance during aging. For this, we performed *in vivo* simultaneous measurements of •NO, O_2 and cerebral blood flow (CBF) in the hippocampus of F344 rats along chronological age in response to glutamatergic activation and in correlation with cognitive performance. Firstly, it is evidenced the temporal sequence of events upon glutamate stimulation of hippocampal *dentate gyrus*, encompassing the local and transitory increase of •NO followed by transitory local changes of CBF and pO_2. Specifically, the transient increase of •NO is followed by an increase of CBF and biphasic changes of the local pO_2. We observed that, although the glutamate-induced •NO dynamics were not significantly affected by aging, the correspondent hemodynamic was progressively diminished accompanying a decline in learning and memory. Noteworthy, in spite of a compromised blood supply, in aged rats we observed an increased ΔpO_2 associated to the hemodynamic response, suggestive of a decrease in the global metabolic rate of O_2. Furthermore, the impairment in the neurovascular coupling observed along aging in F344 rats was mimicked in young rats by promoting an unbalance in redox status toward oxidation via intracellular generation of superoxide radical. This observation strengthens the idea that oxidative stress may have a critical role in the neurovascular uncoupling underlying brain aging and dysfunction. Overall, data supports an impairment of neurovascular response in connection with cognition decline due to oxidative environment-dependent compromised •NO signaling from neurons to vessels during aging.

Keywords: aging, nitric oxide, neurovascular coupling, neurometabolism, oxidative stress, hippocampus

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INTRODUCTION

The brain is an organ with high metabolic and energetic requirements, and despite representing only 2% of the total body weight, the adult brain receives over 15% of the cardiac output and consumes more than 20% of the body's total glucose and oxygen sources (Rolfe and Brown, 1997). Further considering the limited reserves and the high energetic demands, it sounds clear that brain function is critically dependent on adequate blood supply and proper delivery of bioenergetic substrates to support its information processing capabilities through neurotransmission (Zlokovic, 2011). As such, the local intensity of neuronal activity continuously determines both the dynamic regulation of cerebral blood flow (CBF) – neurovascular coupling – and utilization of glucose and O_2 by different cell types – neurometabolic coupling (Ledo et al., 2017). The functional deterioration of these mechanisms is recognized as a pathophysiological feature in brain aging and several age-related neuropathological conditions, such as Alzheimer's and Parkinson's disease (Girouard and Iadecola, 2006; Lourenço et al., 2015; Iadecola, 2017). In addition to the observed inverse correlation between resting CBF and chronological age (Fisher et al., 2013; Desjardins et al., 2014; Tarumi and Zhang, 2017), several studies support the occurrence of reduced hemodynamic responses coupled to neuronal activation during non-pathological aging, both in animals and humans (Park et al., 2007; Gauthier et al., 2013; Fabiani et al., 2014; Toth et al., 2014; Balbi et al., 2015; Jessen et al., 2015; Lourenço et al., 2017). Although a matter of debate, it has also been suggested that cerebral energy metabolism (including both the transport and utilization of oxygen and glucose) is altered during aging (Yin et al., 2016).

Nitric oxide ($^{\bullet}$NO) is a free radical signaling molecule produced by a family of enzymes – nitric oxide synthase (NOS) - that catalyze the conversion of L-arginine to L-citrulline and $^{\bullet}$NO using O_2 and NADPH (Alderton et al., 2001). In the brain, it can be produced within the neurovascular unit either by neuronal, glial or vascular cells, being implicated in several physiological mechanisms, such as synaptic plasticity, modulation of neurotransmitter release, and regulation of CBF (Dawson and Dawson, 1998). In particular, the $^{\bullet}$NO produced by the neuronal isoform of NOS (nNOS) upon stimulation of glutamatergic neurotransmission plays a pivotal role in neurovascular coupling in the hippocampus, acting as a direct signaling messenger from active neurons to blood vessels, thus promoting vasodilation (Lourenço et al., 2014b). A key tenet of $^{\bullet}$NO bioactivity is that besides participating in important physiological functions, it can has also be involved in several pathological mechanisms that actively contribute to neurodegeneration in aging and age-related neuropathological conditions (Yuste et al., 2015). This deviation in $^{\bullet}$NO bioactivity may occur as a consequence of a shift in the redox environment toward more oxidizing conditions. These conditions foster the generation of reactive oxygen and nitrogen species (RONS) capable of interacting with a wide range of molecules, modifying several signaling pathways and ultimately leading to cell damage (Knott and Bossy-Wetzel, 2009).

While several studies have demonstrated age-dependent alterations in $^{\bullet}$NO signaling, CBF, brain metabolism and cognitive decline, the putative connection and the temporal pattern between these factors still requires demonstration. In this work, we aimed to investigate whether brain aging is accompanied by impairment in neurovascular and neurometabolic coupling in the rodent hippocampus, exploring the correlation with learning and memory performance and the glutamate-$^{\bullet}$NO signaling pathway. Furthermore, we explored the putative role of increased oxidative stress in contributing to the neurovascular uncoupling. To this purpose, we performed a cross-sectional study in a rodent model of aging, encompassing behavior testing of cognitive performance and *in vivo* simultaneous measurements of $^{\bullet}$NO, O_2 and CBF upon glutamatergic activation in the hippocampus (*dentate gyrus*) of young, middle- and old-aged Fischer 344 rats.

MATERIALS AND METHODS

Chemicals and Solutions

All chemicals used were analytical grade and obtained from SigmaAldrich, unless otherwise stated. Phosphate buffer (0.05 M PBS) was prepared in MilliQ water and had the following composition (in mM): 10 NaH2PO4, 40 Na2HPO4, and 100 NaCl (pH 7.4). A saturated $^{\bullet}$NO solution was prepared in a Vacutainer® containing MilliQ water purged with Nitrogen (AirLiquid) and then bubbled for 30 min with $^{\bullet}$NO (AirLiquid) gas pre-cleaned by passage in NaOH pellets and 5M NaOH solution, essentially as previously described (Barbosa et al., 2008). The final concentration of this solution has been determined to be 1.8 mM (Zacharia and Deen, 2005). A saturated O_2 solution was prepared in a vacutainer containing PBS Lite bubbled for 30 min with Cabox ($95\%O_2/5\%CO_2$ gas mixture, Linde) as described previously (Lourenço et al., 2017). In accordance to Henry's Law, the final concentration of O_2 at room temperature is 1.3 mM (Sander, 1999).

Animals

All the procedures used in this study were performed in accordance with the European Union Council Directive for the Care and Use of Laboratory animals, 2010/63/EU and approved by local ethics committee (ORBEA). Experiments were conducted in male Fischer 344 rats acquired from Charles River Laboratories (Barcelona, Spain) at 2 months of age and maintained in the animal house facilities of the Center for Neuroscience and Cell Biology (Coimbra). Animals were randomly separated into 3 groups: young (4–6 months old), middle aged (12 months old) and old aged (18 to 23 months old). They were housed in pairs in filter-topped type III Makrolon cages allocated in a room with controlled environment: a temperature of 22–24°C, relative humidity of 45–65%, 15 air exchanges per hour and a 12:12 light/dark cycle. Animals were fed a standard chow rat diet (4RF21-GLP Mucedola, SRL, Settimo Milanese, Italy) and were provided chlorinated water *ad libitum*. Cage bedding (standard corn cob) was changed three times a week and

environmental enrichment was provided with tissue paper and a cardboard tube. The experiments dedicated to the impact of oxidative stress in the neurovascular coupling were conducted in young Wistar rats (3–4 months old) maintained in the same conditions.

Behavior Testing of Memory Performance

The spatial reference memory version of the Morris water maze was performed in a large circular tank (1.5 m diameter, 50 cm depth) filled with water maintained at 22°C. A transparent Plexiglass platform (10 cm diameter) was submerged 1 cm beneath the water surface and maintained in a constant position (centered in an imaginary quadrant). Distinctive visual cues were set up on the wall surrounding the tank, positioned in the midpoint of the perimeter of each quadrant. The training session consisted of 5 consecutive trials during which rats, following a pseudo-random order, were placed in the water facing the tank at the points defined by the quadrants limits. Animals were allowed 60 s to locate the hidden platform, and in case of failure, were guided by the investigator to the platform. In either case, animals were left on the platform for 10 s before being removed from the tank. The latency to find the platform was recorded for each trial. The retention of the spatial training was then assessed after 24 h in a single probe trial consisting of a 60 s free swim in the tank without the platform. The number of crossings over the platform location and the percentage of time spent in the quadrant opposite to the target quadrant was determined as an index of memory performance.

In Vivo Setup for Simultaneous Measurement of •NO, O_2 and Cerebral Blood Flow

The •NO, O_2 and CBF dynamics were measured essentially as previously described (Lourenço et al., 2014b). Both •NO and O_2 measurements were performed using modified carbon fiber microelectrodes (30 μm Ø fiber, Textron Lowell, MA, United States). •NO microelectrodes were modified with Nafion® and *o*-phenylenediamine to improve their analytical properties for •NO *in vivo* measurements. Each sensor was evaluated for sensitivity and selectivity against major interferents (ascorbate, nitrite and dopamine) by amperometry at +0.9 V vs. Ag/AgCl/KCl 3M. For O_2 measurements, carbon fiber microelectrodes were coated with a composite film of multi-wall carbon nanotubes (10 mg/ml) in 0.5% Nafion® and their sensitivity was evaluated by amperometry at −0.55 V vs. Ag/AgCl/KCl 3M. All electrochemical recording were performed using a FAST16mkII bipotentiostat (Quanteon, LLC, Nicholasville, KY, United States) in a two-electrode configuration, as previously described (Barbosa et al., 2008). CBF was measured using a laser Doppler flowmeter device (Periflux system 5000, Perimed, Sweden) coupled with a needle probe (PF411; outer diameter, 450 μm; fiber separation, 150 μm; wavelength, 780 nm). The •NO and O_2 microsensors and the Laser Doppler probe were assembled to an ejection micropipette

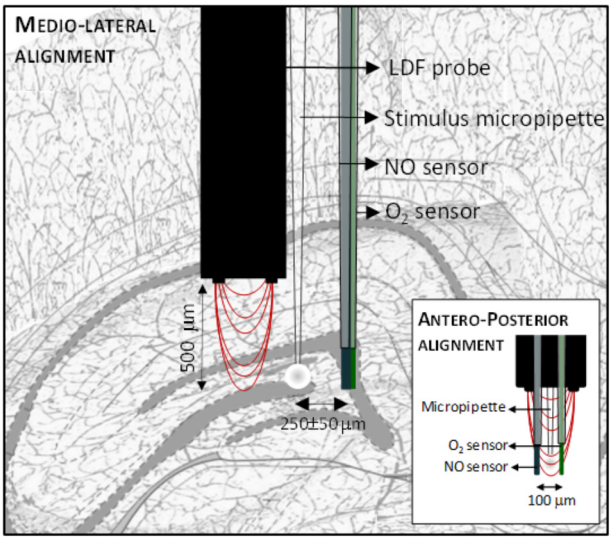

FIGURE 1 | Schematic representation of the array used to simultaneously measure in •NO, Cerebral Blood Flow and O_2 in the *dentate gyrus* of the hippocampus. The array comprises a microsensor for •NO, a microsensor for O_2, a microinjection pipette and a laser Doppler flow probe assembled in the represented geometry.

using sticky wax in a predefined geometric configuration as represented in **Figure 1**. The micropipette was filled with 20 mM L-glutamate prepared in NaCl 0.9% using a syringe fitted with a flexible microfilament (MicroFil, World Precision Instruments, United Kingdom) prior to insertion into the brain.

Rats were anesthetized with isoflurane (induction at 5% and maintenance at 1.5 – 2%) carried in medicinal oxygen (Conoxia, Linde) using an E-Z Anesthesia vaporizer (Braintree Scientific, Inc., United States). Following induction, the animal was placed in a stereotaxic apparatus and body temperature was maintained at 37°C with a heated pad coupled to a Gaymar Heating Pump (Braintree Scientific, Inc., United States). A midline incision was made with a scalp, the skin was reflected and a hole was drilled through the skull, exposing the brain surface overlying the hippocampus. Another hole was drilled in a site remote from the recording area for insertion of a pseudo-reference electrode obtained by produced by electro-oxidation of the exposed tip of a Teflon-coated Ag wire (200 μm o.d., Science Products GmbH, Hofheim, Germany) in 1M HCl saturated with NaCl. After removing the dura matter, the array was inserted into the hippocampus according to coordinates calculated based on the rat brain atlas (Paxinos and Watson, 2007). After the insertion of the array into the hippocampus, it was allowed to stabilize for 20 min. Glutamate was locally delivered by pressure ejection (1 s, 7–15 psi) using a Picospritzer III (Parker Hannifin Corp., General Valve Operation, United States).

To address the impact of oxidative stress in the neurovascular coupling in young Wistar rats, DMNQ (2,3-dimethoxy-1,4-naphthoquinone) was administered by intracerebroventricular injection (200 nmol in 5 μL of saline) between consecutive

glutamate stimulations and the effects evaluated after 30 min, as previously described (Lourenço et al., 2014b).

Protein Blotting Analysis

Dissected hippocampi were homogenized in a potter S homogenizer (2 mL capacity) in 0.4 mL of buffer containing NaCl 150 mM, NP40 1% (v/v), sodium deoxycholate (DOC) 0.5% (w/v), sodium dodecyl sulphate (SDS) 0.1% (w/v), Tris-HCl 50 mM (pH 7.4) and protease inhibitor cocktail 0.1% (v/v). Homogenates were centrifuged at 14000 rpm for 15 min at 4°C and the protein content of the supernatants was quantified using the Bradford Bio-Rad Protein Assay (Bio-Rad, Portugal). Protein extracts (40 μg of protein) were fractionated onto 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, United Kingdom). Western blots were probed with rabbit anti-nNOS polyclonal antibody (Chemicon, Millipore, United States) overnight at 4°C. The bound antibody was detected by alkaline phosphatase-conjugated secondary anti-rabbit antibody (Abcam, United Kingdom) (1/20000 in TBS-T with 1% BSA, 1 h, room temperature), revealed using an enhanced chemifluorescence (ECF) kit (GE Healthcare, United Kingdom) and visualized in a VersaDoc 3000 (Bio-Rad, Portugal). β-Actin was used as control for protein loading.

Data Analysis

The $^{\bullet}NO/O_2$ and CBF recordings were synchronized using OriginPro 7.5 based on markers extracted from the respective recordings. The $^{\bullet}NO$ were characterized in terms of peak amplitude of the signal, based on the conversion of the amperometric currents to fluxes according to Faraday's law (I = n.F.Φ, in which I corresponds to the amperometric current, n corresponds to the one electron per molecule exchanged for the oxidation of $^{\bullet}NO$, F corresponds to the Faraday constant and Φ is the flux). The O_2 dynamics were characterized in terms of maximal amplitude of decrease and increase phases of the glutamate-induced response, based on the conversion of the amperometric currents to pO_2 according to microsensors sensitivity. The CBF changes were characterized in terms of amplitude change (relative to pre-stimulation CBF basal levels). To perform the cross-correlation analysis, the time series of O_2 and CBF recordings were matched and the phase difference between the time courses was characterized by the time corresponding to the maximum of the cross-correlation coefficient in each period. A linear correlation analysis was used to estimate the correlation between the increase in $^{\bullet}NO$ and CBF or between CBF and O_2. For protein blotting analysis, the relative intensities of protein bands were analyzed using the Image J Software. All data are presented as mean ± SEM. Statistical analysis of the data was performed using the GraphPad Software applying ordinary one-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni Multiple Comparison Test or Kruskal–Wallis test followed by Dunn's multiple comparisons test. The statistical significance was considered at $p < 0.05$.

RESULTS

F344 Rats Show Age-Dependent Decline in Spatial Memory

The Morris water maze test was used to assess decline in spatial reference memory, a form of working memory associated with hippocampal function (Vorhees and Williams, 2006). As expected, F344 rats showed decline of both learning and memory capability along age progression, as evidenced by the score of the parameters evaluated both in the acquisition test and in the 24-h retention probe trial (**Figure 2**). In the acquisition test, young and middle-aged animals showed the expected learning curve, with progressive decrease in escape latency is successive trials, while comparatively the old aged animals displayed a delay in learning the localization of the hidden platform (**Figure 2A**). The ability to remember the previous platform location 24-h later was also significantly affected by aging, as reflected by the increase in time spent in the quadrant opposite to the target quadrant and the lower crossings over the platform location. Middle and old aged rats spent an increasing time in the opposite quadrant as compared to young animals (24 and 47%, respectively) (**Figure 2B**). Also, we observed a decrease in the number of crossings over the platform location for the middle and old ages groups when compared to the young group (35 and 61% respectively, **Figure 2C**). These results point toward a progressive age-dependent impairment of spatial reference memory.

The Coupling Between Nitric Oxide Dynamics, Cerebral Blood Flow and pO_2 Is Impaired With Aging in F344 Rats

Measurements of CBF, $^{\bullet}NO$ and O_2 were made simultaneously in the *dentate gyrus* of the hippocampus of anesthetized F344 rats at different ages in response to glutamate stimulations. In all groups, the local injection of glutamate induced transitory changes in $^{\bullet}NO$, CBF, and O_2 with predictable temporal correlation between the three events (**Figure 3**). Specifically, the glutamate-induced increase in $^{\bullet}NO$ was followed by a transient increase in CBF, consistent with the role of neuronal-derived $^{\bullet}NO$ as a mediator of neurovascular coupling in this brain region (Lourenço et al., 2014b). It is of note that, following $^{\bullet}NO$ increase, biphasic changes in the local pO_2 were characterized by an initial decrease matching the time course of $^{\bullet}NO$ dynamics and a later increase matching the time course of CBF increase (**Figure 3B**).

Quantitatively, the peak [$^{\bullet}NO$] resulting from glutamate stimulation showed a tendency to decline with age (28% old vs. young, $p = 0.432$) (**Figure 4A**). In turn, the $^{\bullet}NO$ signal showed slower kinetics in older animals as compared to young animals, reflected in a significant increased signal half-width (43% old vs. young, $p = 0.015$) (**Figure 4B**). This quantitative profile of $^{\bullet}NO$ concentration gradients in the *dentate gyrus* occurs along with a slight increase in nNOS expression levels in the whole hippocampus in old aged animals (20% old vs. young, $p = 0.038$) (**Figure 4C**). Conversely, the hemodynamic response coupled to the $^{\bullet}NO$ dynamics decreased progressively with aging, as shown in **Figure 4D** (71% old vs. young, $p < 0.001$).

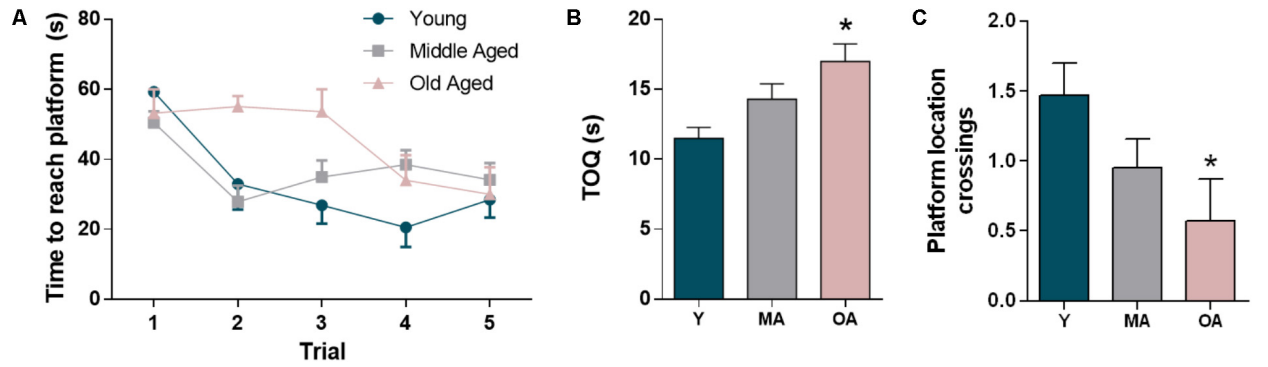

FIGURE 2 | Evaluation of age-dependent decline in short-term memory in the F344 rats. The memory impairment is evidenced by the escape latencies in the training trials **(A)** and the percentage of time spent in the quadrant opposite to the target quadrant **(B)** and the crossings in the platform location **(C)** in the 24-h retention probe test. All the values represent the mean ± SEM. *$p < 0.05$.

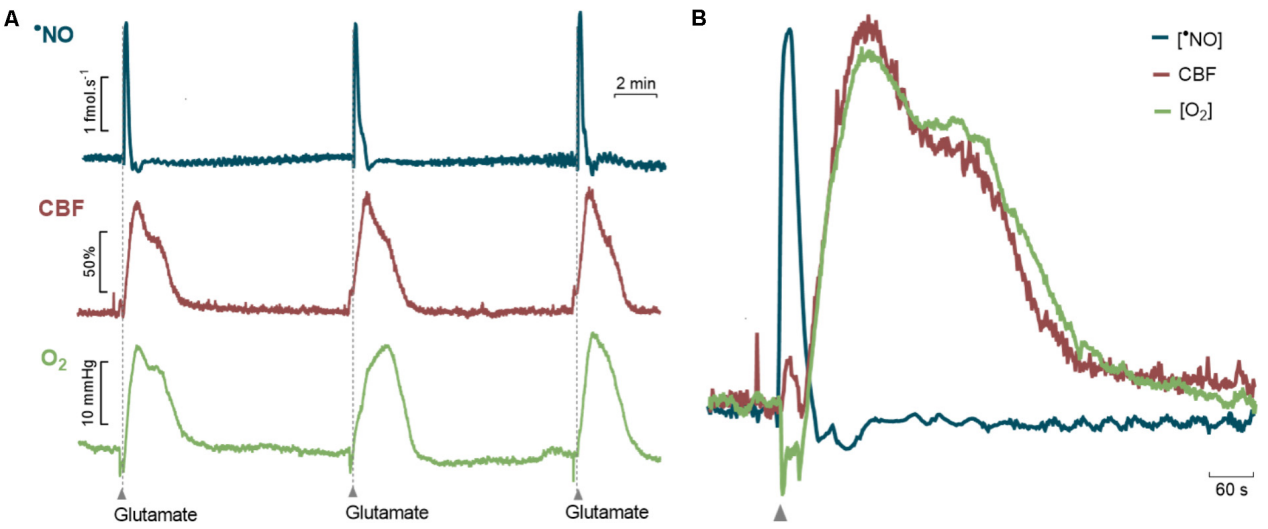

FIGURE 3 | Glutamate-induced changes in •NO, Cerebral Blood Flow and O_2 in the *dentate gyrus* of the hippocampus of anesthetized F344 rats. **(A)** Representative recording of amperometric signals of •NO (blue line), cerebral blood flow (red line) and amperometric signals of O_2 (green line) during glutamatergic activation. Glutamate (0.5 nmol, 1 s) was locally applied by pressure ejection at the time indicated by the upward arrows. **(B)** Detailed temporal correlation between the dynamic changes in the •NO concentration, CBF and O_2 tension.

In aged animals, the impaired CBF change is characterized by an increase in the delay period between •NO rise and onset of CBF response (**Figure 4E**). Noteworthy, the linear correlation between •NO production and CBF observed in young animals is lost for the older animals (R^2 = 0.428, p = 0.021 in young animals; R^2 = 0.267, p = 0.234 in old aged animals) (**Figure 4F**).

Age also influenced the dynamic changes of local pO_2 resulting from glutamatergic stimulation in the hippocampus, in particular the second phase of the signal that appeared temporally correlated with the hemodynamic response (**Figure 5**). While the amplitude of ΔpO_2 during the initial decrease phase, temporally coupled to the transient increase in •NO, remained similar in the three age groups (**Figure 5A**, p = 0.574), the second phase of ΔpO_2 increased significantly in old aged animals (**Figure 5B**, p = 0.010), despite the decrease in amplitude of the CBF changes observed at this age. As shown in **Figure 5C**, and as expected, this results in a significant increase of the ratio between ΔpO_2 and ΔCBF in old-aged F344 rats as compared to younger animals (p = 0.038). Apparently, in the brain of old animals, O_2, although available, is not used as efficiently as in younger animals. In addition to the quantitative changes, the profile of ΔpO_2 was characterized by an increased delay relative to the hyperemic response. The cross-correlation analysis of the time course of ΔpO_2 and CBF dynamics elicited by glutamate evidenced a temporal shift between ΔpO_2 and CBF dynamics in old animals: the average strongest correlation was found at 2 s for young rats (R^2 = 0.917) and at 9 s for old-aged rats (R^2 = 0.813) (**Figure 5D**).

Overall, these results point toward an imbalance in the regulation of both neurovascular and neurometabolic coupling during normal aging, in close correlation with the compromised cognitive function.

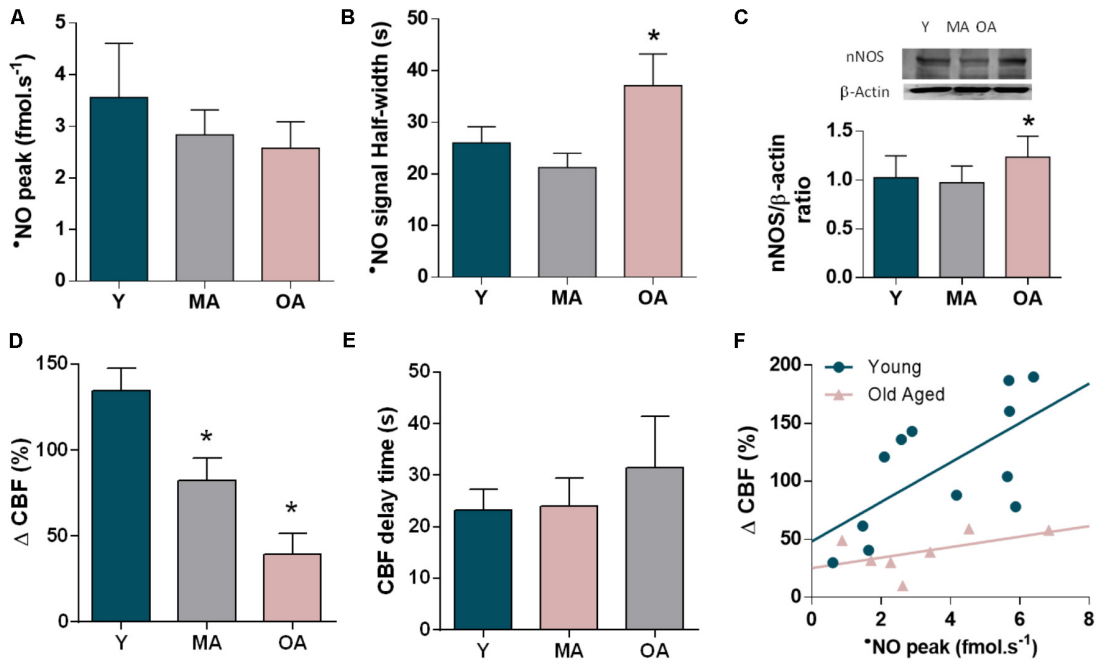

FIGURE 4 | Changes in the •NO-mediated neurovascular coupling along age progression in the *dentate gyrus* of hippocampus of F344 rats. **(A)** Peak amplitude and **(B)** signal duration of the •NO concentration dynamics elicited by glutamate in young (Y), middle aged (MA), and old aged (OA) F344 rats. **(C)** Western Blot of nNOS in hippocampus of Y, MA and OA rats and quantitative analysis of nNOS normalized to β-actin levels. **(D)** Amplitude of the CBF changes coupled to •NO concentration dynamics along age and **(E)** delay period for CBF increase following •NO rise. Each bar represents mean ± SEM. *$p < 0.05$. **(F)** Correlation between the amplitudes of •NO and CBF changes in young (blue) and old aged (pink) F344 rats ($R^2 = 0.428$, $p = 0.021$ in young animals; $R^2 = 0.267$, $p = 0.234$ in old-aged animals).

The Age-Related Uncoupling Between Nitric Oxide Dynamics and Cerebral Blood Flow Is Mimicked by Oxidative Stress Conditions

Oxidative stress has been associated to aging by contributing to the dysfunction of many physiological processes required for proper brain function and to cognitive decline (Perluigi et al., 2014). Hypothesizing that oxidative stress may underlie the observed neurovascular uncoupling during normal aging, we evaluated the effect of increasing the oxidative load on neurovascular coupling in young Wistar rats (**Figure 6**). To test this effect, we used DMNQ, a redox-cycling quinone that triggers intracellular production of superoxide radical (Brunmark and Cadenas, 1989). DMNQ promoted no significant change in the amplitude of glutamate-induced •NO concentration dynamics (**Figure 6A**, $p = 0.592$), but a significant decrease in the coupled CBF changes was observed (**Figure 6B**, 40 ± 14%, $p = 0.038$). Furthermore, we observed a tendency for an increase in the delay period between •NO rise and onset of CBF change following DMNQ as compared to the control signals (**Figure 6C**, $p = 0.063$).

DISCUSSION

In the present study, we sought to investigate the functionality of neurovascular and neurometabolic coupling in association to cognitive performance during aging in F344 rats. We have performed simultaneous measurements of •NO concentration dynamics, pO_2 and CBF responses associated to glutamatergic activation in the *dentate gyrus* of the hippocampus at three different ages. Our findings revealed age-dependent changes in the dynamic profile of •NO, CBF and pO_2 associated with a decline in learning and memory function evaluated by a spatial reference memory version of the Morris water maze test. While only subtle changes were found in glutamate-elicited •NO concentration dynamics, CBF and pO_2 responses were substantially different between young and old aged rats. Interestingly, we found that although the hemodynamic response coupled to the •NO transients showed an age-dependent decline, this was accompanied by an increase ΔpO_2 for this hyperemic phase of the glutamate-evoked response, suggesting a deficient utilization of O_2 in the aged brain.

We have previously described age-dependent changes in endogenous •NO concentration gradients linked to glutamatergic activation, characterized by differences in both the amplitude and temporal profile of the •NO transients in different brain regions (Ledo et al., 2015). Curiously, in the hippocampus, the trend observed in the *dentate gyrus* does not exactly match that previously reported for *CA1* subregion. In particular, while an age-dependent decline in both amplitude and signal duration was reported for *CA1*, here in the *dentate gyrus* we observed an age-dependent increase in the duration of •NO transients in spite of the lower amplitude for the

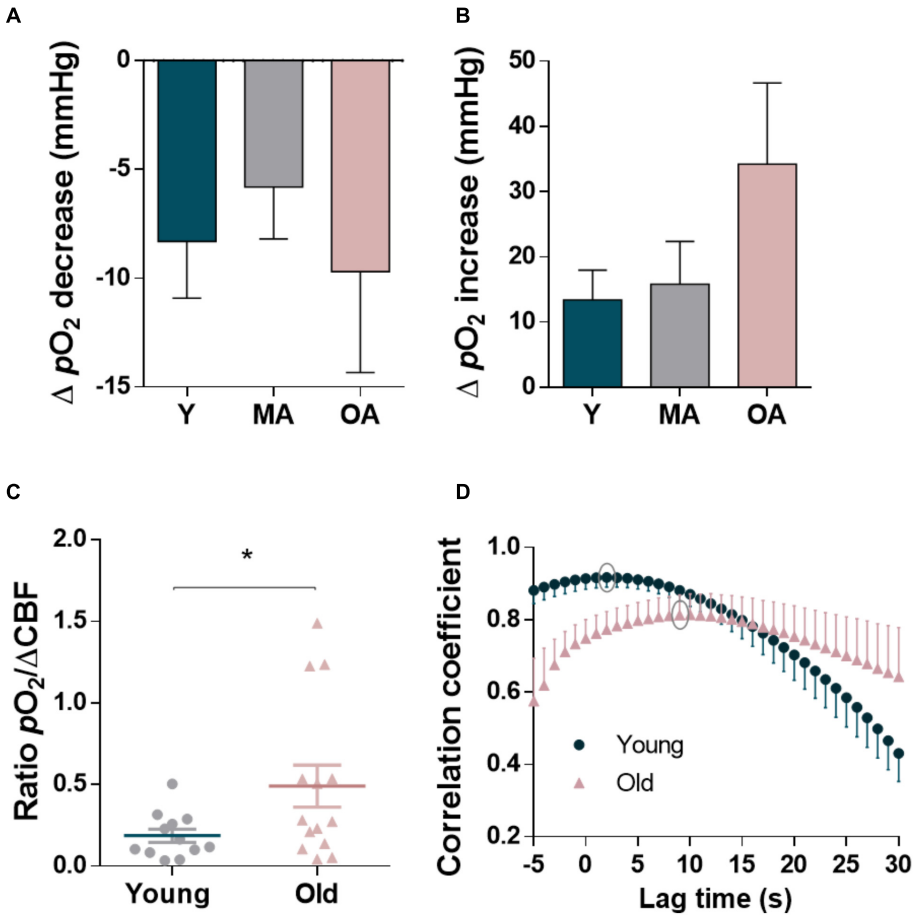

FIGURE 5 | Quantitative analysis of biphasic changes in oxygen tension coupled to the glutamatergic activation in the dentate gyrus of hippocampus of F344 rats along aging and relationship with cerebral blood flow changes. **(A)** Average decrease in ΔpO_2 in the initial component of the signal and **(B)** average increase in ΔpO_2 in the later phase coupled to the CBF increase. Each bar represents mean ± SEM. *$p < 0.05$. **(C)** Ratio of the pO_2 change to the CBF change in young and old-aged animals. **(D)** Cross-correlation analysis of the time course of CBF and O_2 dynamics elicited by glutamate in young and old-aged animals. Average strongest correlation (grey circles) was found at 2 s and 9 s delay of O_2 relative to CBF in young and old rats, respectively. All the values represent the mean ± SEM. *$p < 0.05$.

same stimulation protocol. The concomitant observation that global nNOS expression levels in the hippocampus is higher in older animals strengths the notion of a very tight and specific regulation of •NO signaling amongst different brain regions, as previously suggested (Lourenço et al., 2014a), and supports that it occurs differently in each brain region with age progression. Also, it reinforces the paramount importance of directly measuring the •NO concentration gradients to infer •NO bioactivity. It is known that the •NO synthesis by nNOS can be modulated by several mechanisms, including the availability of cofactors and substrates, interactions between the enzyme with other proteins, subunit dimerization, post-translational modification (Zhou and Zhu, 2009), as well as by oxidative stress conditions (Sun et al., 2008). For instance, in the hippocampus there is evidence for age-related and subregion-specific changes in the activity of arginase, an enzyme that shares the substrate L-arginine with NOS and has a critical role in the regulation of •NO production (Liu et al., 2003; Gupta et al., 2012). Also, several antioxidant defense mechanisms are suggested to be compromised in aged brain in a region-specific manner (Cardozo-Pelaez et al., 1999; Siqueira et al., 2005).

Conversely to the profile of •NO concentration gradients associated to the glutamatergic activation, the •NO-mediated neurovascular coupling deteriorated significantly with age, as revealed by the significantly decreased amplitude and increased delay of the hemodynamic responses in older animals. This observation corroborates several lines of evidence that support the derailment of neurovascular coupling during non-pathological aging in several animal models (Park et al., 2007; Toth et al., 2014; Balbi et al., 2015; Jessen et al., 2015; Lourenço et al., 2017) as well as in humans (Gauthier et al., 2013; Fabiani et al., 2014). Interestingly, in F344 rats the notorious impairment in neurovascular coupling precedes a significant decline in cognitive function, thus providing support for a prominent role of cerebrovascular dysfunction in the deterioration of neuronal function, as previously demonstrated in a mouse model of Alzheimer's disease (Lourenço et al., 2017).

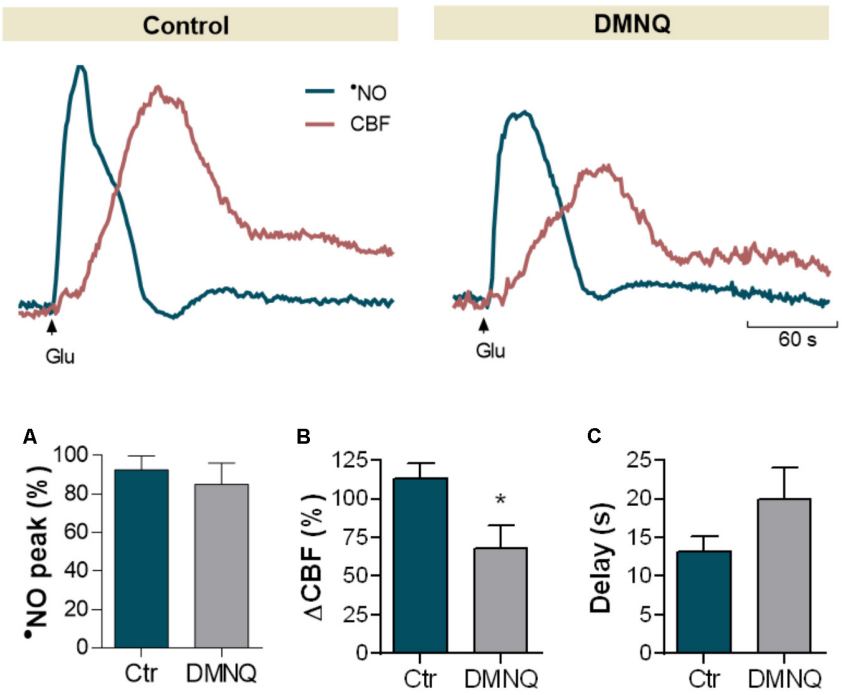

FIGURE 6 | Effect DMNQ, a redox-cycling agent that promotes the intracellular production of superoxide radical, on glutamate-induced •NO signals and CBF changes in the hippocampus of Young Wistar rats. Quantitative analysis of the effect of DMNQ in •NO peak amplitude **(A)**, amplitude in CBF changes **(B)** and delay period for CBF increase following •NO rise **(C)**. Each bar represents mean ± SEM. $^*p < 0.05$.

The age-dependent neurovascular uncoupling here reported also preceded the impairment in the tissue oxygen utilization, as suggested by the observed changes in the glutamate-evoked responses in tissue pO_2 along aging. The temporal profile of tissue ΔpO_2, reflects a moment-to-moment balance between local consumption and supply and the biphasic nature of the signals is supported by the prevalence of each one of these processes in a certain phase: an initial negative component results from the local increase in the rate of O_2 consumption while the later positive component is a result of the increased supply over demand as a result of the local CBF response (Thompson et al., 2003). We observed that the initial component of ΔpO_2 associated with increased O_2 consumption remained unaltered during aging, while the component of the ΔpO_2 correlated with the hyperemic response was significantly increased. This occurred in spite of the compromised CBF response in aged rats, thus suggesting that it is unrelated to increased O_2 delivery but instead with a decrease in the global metabolic rate of O_2. Indeed, by using high resolution respirometry in intact hippocampal slices we have previously demonstrated that both basal and maximal O_2 consumption rates decrease in an age-dependent manner in mice (Dias et al., 2016). Cadenas and coworkers have also demonstrated a reduction in oxidative phosphorylation in F344 rats with aging, linking the impairment of mitochondrial bioenergetics with increased nNOS expression and nitration of relevant mitochondrial proteins (Lam et al., 2009). One would expect that an age-dependent impairment in hippocampal oxidative metabolism be reflected in the two components of the tissue pO_2 dynamics. Yet, this hypothesis cannot be disregarded considering the dynamic nature of the O_2 response and the likely interaction of both components. For instance, in the young animals the smaller delay in the CBF response may reduce the amplitude of the measured initial dip in pO_2. Also, assuming the age-dependent reduction in O_2 metabolism, and thus a higher tissue basal pO_2, we would expect a differential profile of the O_2 consumption related to neuronal activation. Of note, mitochondrial respiration can be decreased in consequence of glutamatergic activation as a result of the reversible and competitive inhibition of with cytochrome *c* oxidase by •NO (Cooper and Giulivi, 2007; Ledo et al., 2010), with the potency of this inhibition being dependent of the O_2 concentration and the enzyme redox state (Mason et al., 2006).

The mechanisms by which aging impairs neurovascular and neurometabolic coupling are likely multifaceted, but the available evidence increasingly supports the primordial role of oxidative stress in age-dependent dysfunction of these and other mechanisms underlying neurodegeneration (Santos et al., 2013; Lourenço et al., 2015; Yin et al., 2016). Previous research supports age-related oxidative damage to several lipids, proteins, and enzymes in F344 rats (Baek et al., 1999; Navarro et al., 2008; Gilmer et al., 2010), in some cases in correlation with changes in mitochondrial bioenergetics (Long et al., 2009) and cerebrovascular dysfunction (Mayhan et al., 2008).

By generating oxidative stress with DMNQ in young Wistar rats we were able to mimic the uncoupling between •NO and CBF dynamics observed in aged F344 rats. This redox-cycling quinone triggers the intracellular production of superoxide that rapidly reacts with •NO in a diffusion-controlled reaction, decreasing •NO bioavailability and generating $ONOO^-$, a potent oxidant (Koppenol et al., 1992; Radi et al., 2002). Although no relevant decrease in •NO dynamics was detected, a significant inhibition of CBF changes was observed under these conditions, suggesting an interruption of the signaling from neurons to vessels. Both the relative abundance of •NO and O_2^- at the source (neurons) and effector site (vascular cells) may contribute to the observed changes. In blood vessels, in addition to a lower •NO concentration in consequence of diffusion, a higher steady-state concentration of O_2^- in association to DMNQ treatment is expected as compared to neurons, due to a lower expression of MnSOD (Ruetzler et al., 2001). This would result in a lower •NO bioavailability at the vessel, thus leading to a reduction in the measured hemodynamic response. A caveat should be made regarding the fact that DMNQ experiments were performed in Wistar rats. In spite of a similar profile in the •NO-dependent neurovascular coupling observed in young Wistar and F344 rats, we cannot disregard the potential interstrain differences in the mechanisms underlying age-dependent neurovascular uncoupling and, as such, warn against the direct transposition of the results between both strains. Yet, in general terms, this observation strengthens the idea that oxidative stress may have a critical role in the neurovascular uncoupling observed in aging as suggested by others. For instance, age-dependent neurovascular uncoupling has been shown to be reversed by interventions that improve oxidative status, including the inhibition of NADPH oxidase (Park et al., 2007) and/or mitochondria-derived production of reactive oxygen species (Toth et al., 2014). This observation with DMNQ encourages further studies addressing the impact of oxidative stress over neurometabolic coupling in correlation to glutamatergic activation. Redox proteomic analysis has revealed oxidative damage of several proteins in the aged rodent brain, many of which involved in bioenergetic biochemical pathways such as glycolysis, the tricarboxylic acid cycle and ATP production (Perluigi et al., 2010). Yet, the impact of these modifications over oxidative metabolism during aging remains controversial (Baek et al., 1999; Navarro et al., 2008; Long et al., 2009; Gilmer et al., 2010; Stauch et al., 2015).

In sum, our study demonstrates that non-pathological brain aging involves changes in both neurovascular and neurometabolic function in the hippocampus, in close correlation with compromised cognitive function, suggesting a role for oxidative stress. This expands our understanding of brain aging and contributes toward a comprehensive elucidation of the mechanisms underlying neurodegeneration in neuropathological conditions for which aging is a major risk factor.

AUTHOR CONTRIBUTIONS

CL contributed to study design, conducted the experiments, analyzed the data, prepared the figures, wrote and revised the manuscript. AL contributed to study design, conducted the experiments, analyzed the data, and revised the manuscript. MC provided assistance to the experiments and data analysis. RB discussed and revised the manuscript. JL conceived and supervised the study and discussed and revised the manuscript. All authors approved the final version of the manuscript.

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REVIEW
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The Role of Oxidative Stress in the Development of Systemic Sclerosis Related Vasculopathy

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Systemic sclerosis (SSc) is a rare connective tissue disease characterized by autoimmunity, vasculopathy, and progressive fibrosis typically affecting multiple organs including the skin. SSc often is a lethal disorder, because effective disease-modifying treatment still remains unavailable. Vasculopathy with endothelial dysfunction, perivascular infiltration of mononuclear cells, vascular wall remodeling and rarefaction of capillaries is the hallmark of the disease. Most patients present with vasospastic attacks of the digital arteries referred to as 'Raynaud's phenomenon,' which is often an indication of an underlying widespread vasculopathy. Although autoimmune responses and inflammation are both found to play an important role in the pathogenesis of this vasculopathy, no definite initiating factors have been identified. Recently, several studies have underlined the potential role of oxidative stress in the pathogenesis of SSc vasculopathy thereby proposing a new aspect in the pathogenesis of this disease. For instance, circulating levels of reactive oxygen species (ROS) related markers have been found to correlate with SSc vasculopathy, the formation of fibrosis and the production of autoantibodies. Excess ROS formation is well-known to lead to endothelial cell (EC) injury and vascular complications. Collectively, these findings suggest a potential role of ROS in the initiation and progression of SSc vasculopathy. In this review, we present the background of oxidative stress related processes (e.g., EC injury, autoimmunity, inflammation, and vascular wall remodeling) that may contribute to SSc vasculopathy. Finally, we describe the use of oxidative stress related read-outs as clinical biomarkers of disease activity and evaluate potential anti-oxidative strategies in SSc.

Keywords: systemic sclerosis, vasculopathy, reactive oxygen species, development, biomarker, intervention

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SYSTEMIC SCLEROSIS

Systemic sclerosis (SSc) is a complex chronic autoimmune disease, characterized by vasculopathy, low-grade chronic inflammation, and fibrosis of the skin and internal organs; the latter causes organ dysfunction, potentially leading to severe morbidity and premature mortality. Vasculopathy is the central feature of the majority of SSc-related complications, for which treatment options are very limited; this has led to a largely unmet medical need. Globally, the incidence rate of

SSc appears to have gradually increased over recent decades, with approximately 7–20 million individuals being diagnosed annually (Nikpour et al., 2010). This increase may be due, in part, to improved diagnosis, but it may also be linked to environmental, nutritional, or other lifestyle-related factors that contribute to the surge in cardiometabolic diseases over the same time span. Systemic sclerosis affects women more frequently than men, and previous reports indicate a slightly higher susceptibility to the disease among African Americans as compared to Caucasians (Laing et al., 1997; Mayes et al., 2003; Ranque and Mouthon, 2010). The two known subtypes (i.e., limited cutaneous SSc and diffuse cutaneous SSc) differ in course and prognosis. The diffuse cutaneous subtype is often associated with the manifestation of devastating complications early in the course of the disease, while such complications occur more insidiously in the limited cutaneous subtype. Although auto-immunity, inflammation, and oxidative stress have all been implicated in the pathogenesis of vasculopathy, the definite factors that initiate this disease remain un-identified (Gabrielli et al., 2009).

Vasculopathy, which affects both small and large blood vessels, is characterized by endothelial dysfunction, perivascular infiltration of mononuclear cells, extracellular matrix remodeling in the vascular wall, and a loss of capillaries (capillary rarefaction) (Fleming et al., 2009; Mostmans et al., 2017). Raynaud's phenomenon (RP) (**Figure 1**), which manifests in the form of recurrent vasospastic attacks that cause episodic discoloration of the extremities in response to cold or emotional stress, is the hallmark of SSc and generally the first detectable sign of the disease (LeRoy and Medsger, 2001; Herrick, 2005; Gabrielli et al., 2009). Following RP, patients may present with critical ischemia and ulceration, which can result in increased morbidity and mortality, as well as a decreased quality of life (Almeida et al., 2015). The SSc-specific abnormalities that occur in the capillaries (e.g., dilatation of capillaries in early stages and loss in later phases) can easily and non-invasively be assessed using nailfold capillaroscopy, which is routinely used in the clinical setting.

There is abundant evidence in the literature that elevated production of reactive oxygen species (ROS)— a collective term for oxygen-derived species with enhanced chemical reactivity— or an unfavorable shift in oxidative/reductive tone in favor of a pro-oxidative milieu (a condition known as "oxidative stress") is involved in a variety of cardiovascular risk factors and diseases (e.g., hypertension, atherosclerosis, and heart failure) (Laursen et al., 1997; Nakamura et al., 1998; Heinloth et al., 2000; Barry-Lane et al., 2001; Hirotani et al., 2002; Tocchetti et al., 2011; Wu et al., 2014). However, little is known about the role played by oxidative stress in the development of SSc-related vasculopathy. Following Murrell's hypothesis (Murrell, 1993) that the pathogenesis of SSc is linked to the generation of ROS, several studies have presented evidence of abnormal ROS production in SSc which, if unabated, may subsequently cause damage (**Figure 2**). Although chronically elevated levels of ROS have been suspected to prolong disease, oxidative stress may also be an initiating factor in the development of SSc vasculopathy. Therefore, a better understanding of the role played by oxidative stress in the development of SSc vasculopathy could help to identify early interventions aimed at delaying the onset of overt symptoms and/or attenuating the course of the disease. In this review, we first describe the physiological role played by ROS in the vascular system. Thereafter, we attempt to explain the role of oxidative stress in the pathogenesis of SSc vasculopathy. Finally, we evaluate the potential of free radical-scavenging therapeutic interventions.

THE ROLE OF ROS IN NORMAL VASCULAR PHYSIOLOGY

Production of Reactive Oxygen Species

Reactive oxygen species were first described by Fenton (1894) and they include free-radical species derived from oxygen such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) and also non-radical oxidants such as hydrogen peroxide (H_2O_2) (Murrell, 1993; Cumpstey and Feelisch, 2017). Reactive oxygen species can be formed non-enzymatically in reactions catalyzed by metals (as in the Fenton reaction); however, in biology, they are mainly generated through intracellular enzymatic sources, and all vascular cell types (including endothelial cells [ECs], smooth muscle cells, and fibroblasts) are able to produce ROS. The majority of ROS are generated during the production of ATP from molecular oxygen, a process also known as mitochondrial respiration (Balaban et al., 2005). During this process, dysregulated mitochondria produce excessive amounts of ROS which can damage all cellular components (Bolisetty and Jaimes, 2013). Mitochondrial electron transport chains consist of four inner-membrane complexes, with the majority of mitochondrial ROS produced by complexes I and III (**Figure 3**) (Berg et al., 2002). Superoxide anion produced by these complexes can be rapidly converted to H_2O_2 by superoxide dismutase (SOD) family of enzymes, comprised of manganese superoxide dismutase (MnSOD) and copper- and zinc- containing superoxide dismutase (Cu,ZnSOD) (Miriyala et al., 2012). Hydrogen peroxide is then converted to water by glutathione peroxidase, or diffused into the cytosol and, thereby, reduces the damaging effect of ROS to the mitochondria (Maharjan et al., 2014). Once ROS is diffused to the cytosol, reactive species are eliminated by the cytosolic antioxidant systems until the cytosolic redox buffer capacity is reached (Maharjan et al., 2014). Also, increased mitochondrial peroxynitrite formation may lead to decreased breakdown of ROS, due to the nitration and inactivation of MnSOD (Daiber, 2010). Furthermore, cytosolic ROS production by NADPH oxidases may trigger mitochondrial ROS formation through several processes. For instance, the opening of the redox sensitive K_{ATP} channels may lead to changes in the mitochondrial membrane potential, and thereby causing a rise in mitochondrial ROS production (Garlid et al., 2003).

Other sources of intracellular ROS production include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzymes (Bedard and Krause, 2007) uncoupled nitric oxide synthases (NOSs) (Landmesser et al., 2003; Taniyama and Griendling, 2003; Turrens, 2003; Kawashima et al., 2007; Nishino et al., 2008) xanthine oxidases and lipoxygenases (Murrell, 1993). In most of these reactions, ROS formation is a by-product of

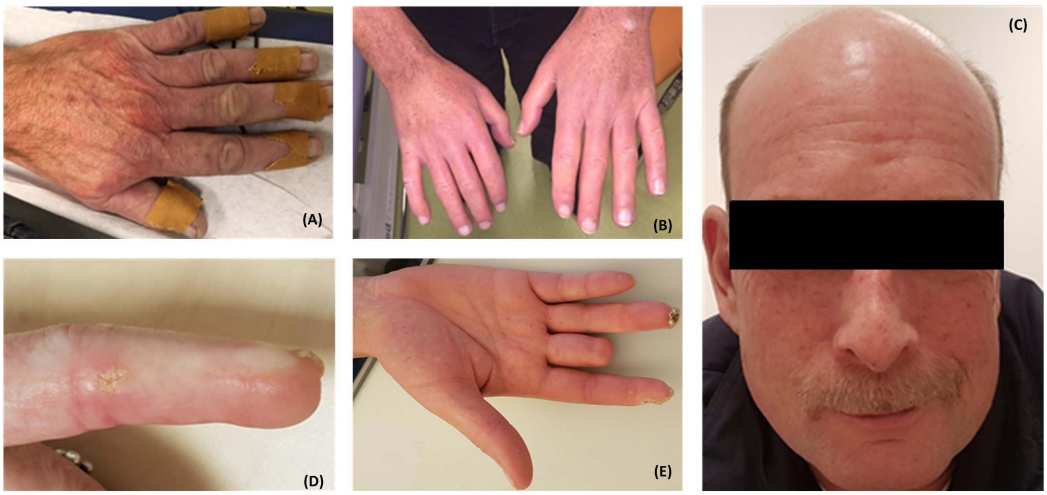

FIGURE 1 | Patients with systemic sclerosis (SSc) often present with Raynaud's phenomenon **(A)**. Patients may subsequently develop tightening and thickening of the skin of the fingers (sclerodactyly) **(B)**, telangiectasia **(C)**, calcinosis cutis **(D)**, and digital necrosis which may lead to amputation of the digits **(E)**. Written informed consent was obtained from the patients for the publication of these images.

oxidative metabolism (and is often considered an undesirable result of electron leakage), although this is clearly not the case for NOX enzymes, whose sole purpose is the production of superoxide for signaling purposes or (at much higher rates of production) microbial killing. Mammals are equipped with antioxidant systems that offer protection against the damaging off-target effects of ROS, and several antioxidants and antioxidant enzymes have been described. The antioxidant buffering systems include low-molecular weight substances such as nutritional antioxidants (e.g., vitamin C and vitamin E) (Benfeito et al., 2013) sulfhydryl (SH)-containing compounds (e.g., thiols such as glutathione) (Turell et al., 2013) and antioxidant enzymes (including SOD, catalase, and glutathione peroxidases) (Matés et al., 1999). An imbalance between the production of ROS and their inactivation by antioxidants and reducing enzymes that results in ROS overproduction is referred to as "oxidative stress" (Betteridge, 2000; Grygiel-Górniak and Puszczewicz, 2014). In response to an increase in endogenous production of ROS, an organism may adapt by increasing its antioxidant capacity (commonly referred to as the redox-buffering capacity). If an organism is unable to maintain the appropriate redox homeostasis, disturbances in this balance can cause harmful effects that can ultimately damage the structural and functional components of a cell, leading to the oxidation of proteins, lipids, and DNA (Harman, 1956).

Reactive Oxygen Species Signaling in Vascular Tone Regulation

Reactive oxygen species serve as important signaling molecules that regulate a variety of physiological processes in order to maintain appropriate redox homeostasis (Jones and Sies, 2015). In the vasculature, for example, ROS have been identified as key signaling molecules involved in the fundamental function of vascular smooth muscle cells (VSMCs), including the ability of blood vessels to contract or relax (MacKay and Knock, 2015). However, the available data underline the complexity of the effects of ROS on the vascular system, as —depending on the conditions— both contraction and relaxation of the vascular muscle may occur (Faraci, 2006). Superoxide anion has a contractile effect on VSMC, and removal of the endothelium prevents this vasoconstriction, making this process endothelium dependent (Katusic and Vanhoutte, 1989). In line with this finding, the toxicant benzo(a)pyrene has been found to decrease the endothelium-dependent NO-induced vasodilation in retinal arterioles through the production of superoxide (Kamiya et al., 2014). Vasoconstriction, as an acute response to ROS, is caused by the scavenging of endothelial nitric oxide ($NO^{\bullet}$), another free radical and a potent vasodilator produced in arterial blood vessels to regulate vascular tone and blood flow (Moncada et al., 1991). Chronically, vasoconstriction may also be caused by an increased expression of contractile proteins. Likewise, ROS production has previously been associated with a twofold increase in the expression of contractile proteins and microRNA (miR-145), which can up-regulate transcription of contractile protein genes (Chettimada et al., 2014). In addition, increased contractility of VSMCs by affecting the Ca^{2+} signaling pathway has previously been described (Touyz and Schiffrin, 2004). Several studies have also reported on the vasodilatory effect of ROS, especially in cerebral arteries. Superoxide, generated by xanthine and xanthine oxidase, produces dilation of cerebral arterioles, presumably mediated through potassium channel opening (Wei et al., 1996). Moreover, overproduction of H_2O_2 has been suggested to lead to vasodilatation through an enhanced endothelial response to acetylcholine (Drummond et al., 2000; Thomas et al., 2002; Zhou et al., 2013). A current hypothesis poses that ROS may have a biphasic response, with lower concentrations resulting in vasodilation and higher rates of

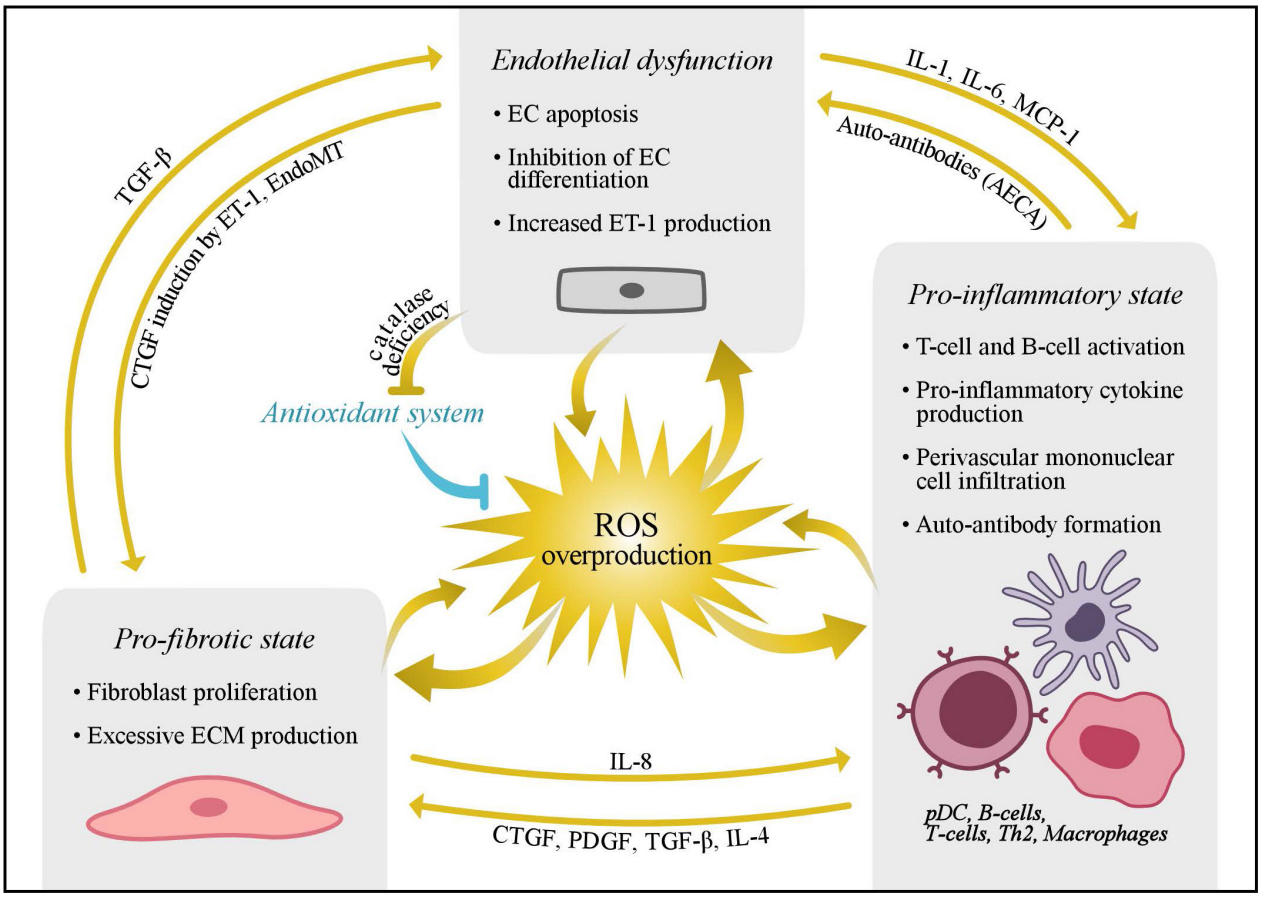

FIGURE 2 | Schematic presentation of the key elements in the pathogenesis of SSc. Overproduction of reactive oxygen species may induce endothelial dysfunction and promote a pro-fibrotic and pro-inflammatory state. Moreover, interplay between these processes has been proposed to further cause damage, and various cytokines and chemokines are thought to play an important role in this process. AECAs, anti-endothelial cell antibodies; CTGF, connective tissue growth factor; ECs, endothelial cells; EndoMT, endothelial to mesenchymal transformation; ECM, extracellular matrix; ET-1, endothelin-1; IL, interleukin; MCP, monocyte chemotactic protein 1; pDC, plasmacytoid dendritic cells; PDGF, platelet derived growth factor; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; Th, T helper cell.

production causing vasoconstriction (Faraci, 2006) potentially explaining the contradictory effects of ROS on the vascular system.

$NO^{\bullet}$, itself a free-radical reactive nitrogen species (RNS), is also suspected of playing a critical role in vascular homeostasis. $NO^{\bullet}$ is a potent activator of soluble guanylyl cyclase, which converts guanosine triphosphate (GTP) to the second messenger cyclic guanosine monophosphate (cGMP), causing vasodilation by increasing calcium handling, which stimulates the contractile function of the VSMC (Arnold et al., 1977). $NO^{\bullet}$ is synthesized from the amino acid L-arginine by a family of isoenzymes termed NOSs. The synthesis of $NO^{\bullet}$ occurs in response to various physicochemical stimuli, including neurotransmitters, shear stress, and growth factors. Furthermore, $O_2^{\bullet-}$ and $NO^{\bullet}$ can readily interact with each other to form peroxynitrite ($ONOO^-$); this reaction accounts for the vasoconstriction observed by enhanced ROS production and explains the vasorelaxant effects of SOD in experiments with isolated blood vessels. Prolonged exposure of ECs to peroxynitrite can lead to oxidation of the NOS cofactor, tetrahydrobiopterin, and protein tyrosine nitration, which may impair NOS function following the uncoupling of electron flow from arginine oxidation; these events ultimately translate into a low NO bioavailability and impaired vasodilatation (Aliev et al., 1998; Dimmeler and Zeiher, 2000). Hydrogen sulfide (H_2S) — a reactive signaling molecule belonging to the group of "gasotransmitters" (Wang, 2002) and a reactive sulfur species (RSSs) (Cortese-Krott et al., 2017) produced by ECs and perivascular adipose tissue—may also promote vasodilation through the activation of endothelial NO synthase, the inhibition of cyclic guanosine monophosphate (cGMP) degradation, and by activating potassium channels in VSMCs (Bełtowski and Jamroz-Wiśniewska, 2014). Because of the propensity of ROS, NO, and H_2S to chemically and functionally interact with each other at multiple levels, Cortese-Krott et al. (2017) have underscored the importance of viewing these reactive species as a unified entity (defined as the "reactive species interactome"), rather than as separate signaling entities.

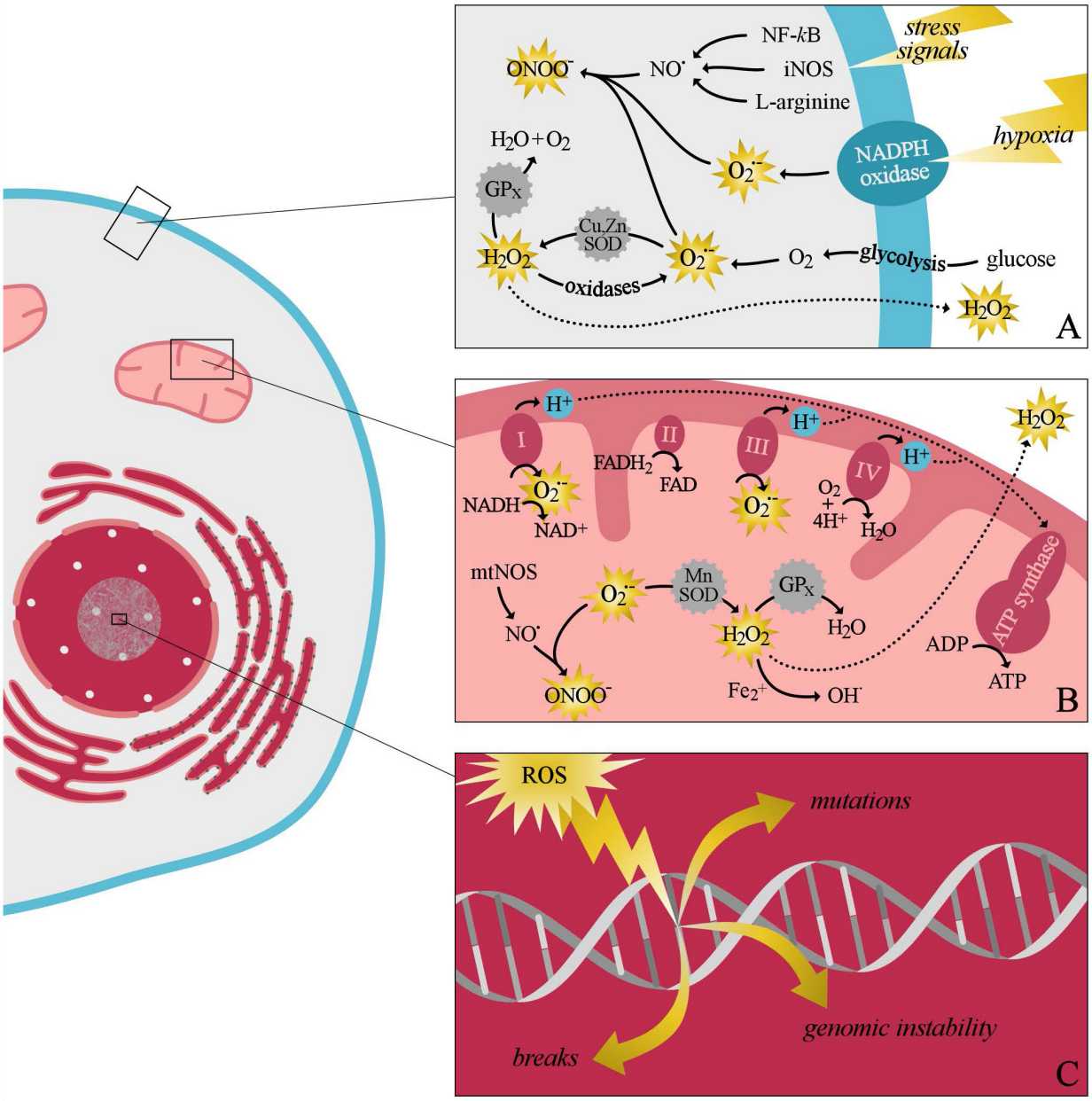

FIGURE 3 | A simplified schematic overview of intracellular production of reactive oxygen species (ROS) and the intracellular antioxidant systems. Cytosolic ROS production can be evoked as a response to several stress signals, including hypoxia **(A)**. During this process superoxide anion is mainly produced by oxidases, including NADPH oxidase, xanthine oxidase, and lipooxygenase. After its production, superoxide is rapidly converted by the antioxidant system to, the less damaging radical, hydrogen peroxide and water. Furthermore, cytosolic ROS production can promote mitochondrial ROS production **(B)**. Superoxide anion produced by the different complexes of the electron transport chain can easily react with nitric oxide to form peroxy nitrite species. Alternatively, superoxide can also be converted by manganese superoxide dismutase (SOD) to hydrogen peroxide. Hydrogen peroxide can be either converted to water by glutathione peroxidase, or diffused to the cytosol. If the production of free radicals exceeds the antioxidants buffering capacity, damage to all component of the cell may occur **(C)**. ATP, adenosine triphosphate; ADP, adenosine diphosphate; FAD/$FADH_2$, flavin adenine dinucleotide; Fe^{2+}, iron; H_2O_2, hydrogen peroxide; H_2O, water; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; iNOS, inducible nitric oxide synthase; NAD^+/NADH, nicotinamide adenine dinucleotide; $NO^•$, nitric oxide; mnSOD, manganese superoxide dismutase; mtNOS, mitochondrial nitric oxide synthase; $O_2^{•-}$, superoxide; $OH^•$, hydroxyl radical; $ONOO^-$, peroxynitrite; Cu,ZnSOD, copper- and zinc-containing superoxide dismutase; GP_x, glutathione peroxidase.

In addition to the direct regulation of vascular tone, ROS are also essential for maintaining O_2 hemostasis by stimulating several transcription factors, such as hypoxia-inducible factor-1 (HIF-1) (Chandel et al., 1998, 2000; Brocato et al., 2014; Friedman et al., 2014). Hypoxia increases ROS production by a variety of mechanisms, triggering marked alterations in redox signaling

(Smith et al., 2017). Furthermore, ROS upregulate vascular endothelial growth factor (VEGF) expression via activation of HIF-1, subsequently leading to angiogenesis (Arbiser et al., 2002; Xia et al., 2007). Although these studies shed light on the possible role of ROS in O_2 sensing, their exact role in these and other mechanisms often remains unclear and awaits further clarification. Of note, H_2S is also suspected of being involved in O_2 sensing (Olson, 2015) and the availability of both H_2S and ROS is modulated by their interaction with NO (Cortese-Krott et al., 2017) as mentioned above.

Reactive Oxygen Species Signaling in Other Cellular Processes and Immunity

Increasing evidence suggests that ROS also play an important role in a variety of cellular activities through several signaling pathways (e.g., the NF-κB signaling pathway and the MAPKs signaling pathway) (Zhang et al., 2016). Several experimental studies have demonstrated that a biphasic effect (i.e., low concentrations increasing the cell number and high concentrations decreasing the cell number) of ROS occur in fibroblasts, cultured smooth muscle cells, cultured ECs, and immortalized lung epithelial cells (Burdon, 1995; Arnold et al., 2001; Day and Suzuki, 2005). Additionally, human embryonic stem cells have previously been found to engage in spontaneous differentiation when they were cultured under normoxic conditions (21% O_2); however, reduction of the oxygen saturation to 1% O_2 inhibits cell proliferation (Ezashi et al., 2005). Hypoxia-driven ROS production could partially explain the described observations. Furthermore, both the innate and adaptive immune systems are thought to be affected by ROS production. For example, it has been reported that ROS play a crucial role in T-cell activation, a process that is also associated with an increase in cell ROS production (Devadas et al., 2002). These findings highlight the importance of ROS in the regulation of several cellular processes.

THE ROLE OF OXIDATIVE STRESS IN SSc VASCULOPATHY

Link Between Oxidative and Endothelial Cell Dysfunction/Injury

The EC is believed to be a main target for pathological processes in SSc (Mostmans et al., 2017) and ROS have been implicated as having an important role in this process. In SSc, oxidative stress may cause the activation and damage of ECs (Grygiel-Górniak and Puszczewicz, 2014) leading to EC apoptosis and impairment of cell–cell adhesion (Sgonc et al., 1996). This impairment ultimately increases vascular endothelial permeability, subsequently leading to alterations in EC signal transduction (Fleischmajer and Perlish, 1980). When damaged, vascular ECs produce increased levels of vasoconstrictive mediators (e.g., endothelin-1 [ET-1]) and show an impaired release of NO and prostacyclin (Freedman et al., 1999; Romero et al., 2000; Matucci Cerinic and Kahaleh, 2002). This imbalance in mediators, in addition to several other oxidative and non-oxidative pathways (e.g., the adrenergic mechanism), results in altered vascular tone in favor of vasoconstriction. Consistent with our hypothesis that oxidative stress plays a crucial role in the development of SSc vasculopathy, ECs from SSc patients were previously reported to have the ability to produce H_2O_2 (Servettaz et al., 2007) which may inhibit endothelial differentiation due to a reduced octamer-binding transcription factor 4 (Oct-4) expression (Xiao et al., 2014). Furthermore, endothelin-1 and angiotensin II (a bioactive peptide of the renin–angiotensin system), which are often elevated in SSc, may upregulate mitochondrial ROS generation through the activation of NADPH oxidase (Nozoe et al., 2008; Wosniak et al., 2009; Wen et al., 2012). In addition to this ability to produce ROS, ECs are also more prone to ROS injury due to a deficiency in catalase synthesis (Jornot and Junod, 1992). This deficiency may initiate a self-perpetuating cycle of recurrent RP attacks, increased ROS production (generated during the conversion of xanthine dehydrogenase to xanthine oxidase), more injured ECs, and an inability to defend against oxidative stress (Herrick and Matucci Cerinic, 2001; Chung et al., 2006; Ogawa et al., 2006; Servettaz et al., 2009). In line with these observations, increased levels of isoprostanes (oxidized lipids and markers of oxidative stress) have been found to correlate with the extent of vascular damage in SSc (Ogawa et al., 2006, 2010).

Although inhibition of EC growth has previously been found to be associated with NO overproduction, in SSc, a paradoxical decrease in NO bioavailability has been observed (Matucci Cerinic and Kahaleh, 2002). This can partially be explained by the rapid reaction of $NO^{\bullet}$ with the superoxide anion $O_2^{\bullet-}$, forming peroxynitrite (Sambo et al., 2001b). With disease progression, the production of eNOS is further downregulated (Bruckdorfer, 2005). As a result of EC injury and subsequent vascular remodeling, SSc patients show specific capillaroscopic patterns consisting of micro-hemorrhages, as well as dilated, giant, and malformed capillaries (Maricq et al., 1980). These nailfold patterns are positively associated with the degree of vasospasm and ischemia, further indicating that ROS plays a role in the occurrence of microvascular injury (van Roon et al., 2016).

Link Between Oxidative Stress and Auto-immunity

Several SSc-specific auto-antibodies (e.g., anti-topoisomerase 1, anti-centromere antibodies, anti-RNA polymerase III, anti-U3-RNP, TH/To RNP, U1-RNP, and PM-Scl) have previously been identified, and ROS are thought to be involved in the process leading to autoimmunity. More recently, subcutaneous injections of agents generating $OH^{\bullet}$ or HOCl (another ROS) in mice induced cutaneous and lung fibrosis, as well as the production of serum anti-DNA topoisomerase 1 antibodies (Servettaz et al., 2009). Furthermore, it has been shown that injections of $ONOO^-$–generating agents induce the production of anti-centromere B antibodies in the absence of serum anti-DNA topoisomerase 1 antibodies. These findings support the hypothesis that bursts of ROS may cause the oxidation of DNA-topoisomerase-1, which may lead to immunological intolerance to this nuclear antigen. In line with these findings, mice exposed

to HOCl developed anti-DNA topoisomerase I antibodies and diffuse cutaneous SSc with pulmonary fibrosis (Kavian et al., 2010).

Autoimmunity may occur through the impairment of DNA repair mechanisms (**Figure 2**) (Birben et al., 2012). For example, it has previously been established that 8-oxo-7 hydrodeoxyguanosine levels in lymphocytes were increased in several rheumatic diseases, as compared to healthy controls (Bashir et al., 1993). More recently, autoantibodies against methionine sulfoxide reductase A, one of the antioxidant repair enzymes, have been detected in 33% of SSc patients (Ogawa et al., 2010). This finding supports the hypothesis that an altered defense mechanism may contribute to the development of vasculopathy. In addition, this finding is also indicative of a promutagenic DNA lesion, presumably induced by ROS and defective DNA repair mechanisms (Bashir et al., 1993).

Recently, several studies have investigated the role of a new class of antibodies that may be directly linked to the pathological processes that occur even in the early stages of SSc. For instance, antiendothelial cell antibodies (AECAs) are thought to play an important role in EC injury promoting the development of vasculopathy (Hill et al., 1996; Bordron et al., 1998; Boin and Rosen, 2007; Mihai and Tervaert, 2010). Anti-endothelial cell antibodies may induce endothelial perturbation, an increase in adhesion molecule expression (ICAM-1, VCAM-1, and E-selectin) and stimulate the secretion of pro-inflammatory cytokines and chemokines (Corallo et al., 2015). The formation of AECAs is thought to be initiated by the exposure of the nuclear contents of damaged ECs (Praprotnik et al., 2001) which may very well be induced by excessive ROS production. Moreover, some evidence provided using a molecular cloning strategy suggests that these antibodies (e.g., AECAs) cause oxidative imbalance and subsequently lead to endothelial apoptosis, suggesting causal involvement in the disease process (Margutti et al., 2012).

Some evidence supports the hypothesis that heat shock proteins (HSP) play an important role in the pathogenesis of SSc (Danieli et al., 1992; Ogawa et al., 2008). Heat shock proteins are a family of immunogenic proteins that are found in all organism cells, and are essential for various cellular function. These proteins have chaperon properties and facilitate in protein folding, assembly, and intracellular transport (Brenu et al., 2013). The synthesis of HSP is increased in response to a variety of stressful stimuli, such as hyperthermia, hypoxia, inflammation, and auto-immunity (Kiang and Tsokos, 1998; Fujimoto et al., 2004). Heat shock proteins have previously been found to stimulate antigen presenting cells, which then activate the adaptive immune cells, suggesting a critical role of HSPs in the immune system (Brenu et al., 2013). Furthermore, increased levels of antibodies to HSP have previously been demonstrated in several immune diseases, including atherosclerosis, type 1 diabetes mellitus and various auto-immune rheumatic diseases (Raska and Weigl, 2005; Shukla and Pitha, 2012). Moreover, the 60-kDa heat shock protein (HSP60) was previously found to be a target antigen for AECAs, and thereby induce apoptosis of ECs in variety of rheumatic diseases, including SSc. In support, serum HSP70 levels are also found to be increased in SSc patients, and are associated with the occurrence of fibrosis, oxidative stress, and inflammation (Ogawa et al., 2008). Moreover, expression of HSP47 was found to be linked to the overproduction of type I collagen in cultured SSc fibroblast (Kuroda et al., 1998). These results shed light on the potential of these markers as serological biomarkers of cellular stress. Conversely, there is some evidence supporting the postulation that HSPs also have a beneficial effect following oxidation (Kalmar and Greensmith, 2009). These effects are likely linked to the sensor ability of redox changes and cytoprotective effects of HSPs. However, the exact role in the pathogenesis of SSc has to be elucidated further.

Age related auto-immunity is also thought to play an important role in SSc. Approximately, 21% of all SSc patients are aged 65 years or older at diagnosis (Pérez-Bocanegra et al., 2010). This observation has instigated great interest in the role of key determinants of biological aging (e.g., telomere attrition, senescent cells) in the pathogenesis of SSc. Autoimmunity in the elderly population is also believed to be promoted by accelerated cellular senescence (Piera-Velazquez and Jimenez, 2014) and through the increased affinity of T-cells to self-antigens (Goronzy and Weyand, 2012). It was previously demonstrated that the age of disease onset strongly influences clinical signs of SSc, the presence organ involvement and outcome. Moreover, the elderly SSc patients, compared to their younger counterparts, were more frequently diagnosed with cardiopulmonary organ involvement (Alba et al., 2014). A study conducted by Shiels et al. (2011) demonstrated that patients diagnosed with dcSSc exhibit features consistent with accelerated biological aging, and this was found to be independent of increasing age. This study concluded that abnormal telomere biology may induce the development clinical signs in SSc patients. Supporting this hypothesis, a previously conducted study found that the telomere length of SSc patients, and their family members, were on average 3 Kb shorter than age-matched controls. In addition, they stated that the telomeric length in SSc patients did not decrease significantly with age (Artlett et al., 1996). This reduction in telomere length can partially be explained by the elevated oxidative stress levels in these patients, which may accelerate telomere erosion (MacIntyre et al., 2008). Moreover, this observation may also be explained by the presence of autoantibodies against telomere binding proteins.

Link Between Oxidative Stress and Inflammation

Chronic ROS overproduction may stimulate a pro-inflammatory state in SSc (Yamagishi et al., 1994; Suzuki et al., 1999). The early phase of SSc is characterized by perivascular mononuclear cell infiltration (Mostmans et al., 2017) and the activation of the innate and adaptive immune system, which results in B-cell and T-cell activation (Amico et al., 2015). These cells secrete pro-inflammatory cytokines (including IL-1, IL-6, and IL-8) and chemokines that further stimulate ROS production (Murrell, 1993; Hua-Huy and Dinh-Xuan, 2015). However, ROS have been reported to promote activation of a variety of inflammation-associated transcription factors (e.g., nuclear factor-kappa B [NF-κB], activator protein-1 [AP-1], p53, signal transducer and activator of transcriptions 3 [STAT3], HIF-1α, and NF-E2 related factor-2 [Nrf2]). This process can further

aggravate the inflammatory response through the production of a variety of inflammatory mediators (Hussain and Harris, 2007). Although these findings emphasize that the development of SSc vasculopathy is due to interconnected pathways of both inflammatory processes, as well as oxidative stress, the level of oxidative stress in SSc seems to exceed that which can be explained solely by the inflammatory process. Therefore, our hypothesis that oxidative stress initiates the development of vasculopathy appears to be reasonable.

Link Between Oxidative Stress and Vascular Wall Remodeling

Vascular remodeling affects a variety of sites, including the lungs, the heart, the skin and the kidneys, resulting in critical luminal narrowing of the vessel (**Figures 4**, **5**). Severe complications, such as pulmonary arterial hypertension (PAH) and scleroderma renal crisis (SRC), are accompanied by this luminal narrowing. The occlusion of the microvasculature due to intimal and media proliferation may result in an impaired tissue supply of oxygen and nutrients, leading to increased ROS production and, eventually, to a loss of vasculature (Fleming et al., 2009). In addition, several signals involved in SSc pathogenesis, including transforming growth factor-beta (TGF-β), PDGF, and ET-1 modulate the expression of NOX, which may lead to the formation of fibrosis by up-regulating ROS production (Eckes et al., 2014).

Reactive oxygen species have been implicated in several SSc-related processes, including the stimulation of fibroblast proliferation, collagen-gene expression, and phenotype conversion of myofibroblast (Sambo et al., 2001b). An imbalance between collagen and elastin and the infiltration of the vessel wall by VSMCs, mononuclear cells, and other inflammatory cells, as well as an increased production of cytokines, MMP, chemokines (MCP-1), cell adhesion molecules, and growth factors (TGF-β, CTGF, PDGF), all contribute to vascular wall thickening. Accordingly, it has been observed that extracellular matrix deposition into the vessel wall results from disproportionate fibroblast activity with increased serum levels of hyaluronan, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) (Montagnana et al., 2007). TGF-β, in particular, promotes the synthesis of collagen, glycosaminoglycans, and fibronectin by fibroblast, and reduces the collagenase-synthesizing capacity of fibroblasts (Smith and LeRoy, 1990).

Perivascular cells including, but not limited to, smooth muscle cells (SMC), and in particular VSMCs, are thought to play a key role in maintaining vascular integrity. During physiological processes, these cells have a contractile function, and thereby regulate blood flow. Current literature suggest that intimal hyperplasia may be formed by migration and proliferation of medial SMC and adventitial fibroblast (Fleming et al., 2009) as a response to various stimuli including oxidative stress. Subsequently, VSMCs produce excessive amount of ECM to form a fibrotic vascular lesion. Moreover, following the initial vascular injury, endothelial progenitor cells are mobilized from the bone marrow, recruited to the site of the vascular lesion and differentiate into a variety of cells, including VSMCs (Dimmeler et al., 2005). Proliferation of VSMCs is usually regulated by NO, which inhibits cell proliferation through upregulation of cGMP. However, due to the impaired NO production in SSc, processes related to the inhibition of cell proliferation may be altered, and ECM production may be promoted further. In addition to the decreased production of NO, cell proliferation is also promoted by upregulation of ET_B receptor on SMCs (Shetty et al., 1993). In addition, migration and proliferation of VSMCs is also likely due to the impaired interaction between ECs and VSMCs. This impaired interaction, which is characterized by a decreased expression of α-SMA in VSMCs, is thought to induce phenotypic modulation in VSMC. This phenotypic modulation induces VSMCs to act in a more in more migratory and proliferative manner (Asano et al., 2010).

Vascular remodeling is also promoted by the ability of ECs to convert into myofibroblast under hypoxic and pro-oxidative milieu, a process known as "endothelial to mesenchymal transition" (Jimenez, 2013). On the other hand, NO has been reported to have an antifibrotic effect in experimental animal models (Yoshimura et al., 2006). NO can directly activate several transcription factors (such as nuclear factor-κB, specificity protein-1, and activator protein) to inhibit collagen gene expression (Bogdan, 2001). In addition, NO mediates prolyl hydroxylase expression, which is important in the post-translational processing of collagen (Dooley et al., 2012). Therefore, the formation of fibrosis may be stimulated by the decreased bioavailability of NO in SSc patients.

HIF-1α is generally increased substantially as a response to low oxygen concentrations, which generally promotes ROS production. This upregulation of HIF-1 controls the expression of several genes involved in processes such as erythropoiesis, angiogenesis, glucose metabolism, cell proliferation, and cell apoptosis (Maxwell and Ratcliffe, 2002; Beyer et al., 2009). Tissue hypoxia usually triggers angiogenesis in order to improve oxygenation of hypoxic tissue. As a response to chronic hypoxia due to progressive loss of vasculature, levels of VEGF often increase. Accordingly, both receptors for VEGF (e.g., VEGF receptors 1 and 2) have previously been found to be overexpressed in the skin and serum of SSc patients (Chora et al., 2017). Reactive oxygen species have been implicated to stimulate the induction of VEGF expression in variety of cells, including ECs, VSMC, and macrophages (Ruef et al., 1997; Wang et al., 2011; Kim and Byzova, 2014). Simultaneously, VEGF further increases the production of ROS through the activation of NADPH oxidase in ECs (Ushio-Fukai and Alexander, 2004).

BIOMARKERS OF OXIDATIVE STRESS

In the past decade, several *in vitro* and *in vivo* studies have demonstrated the role played by the overproduction of free radicals in the pathophysiology of SSc, and several biomarkers of this process have been described. These biomarkers include oxidized low-density lipoprotein, malondialdehyde (MDA),

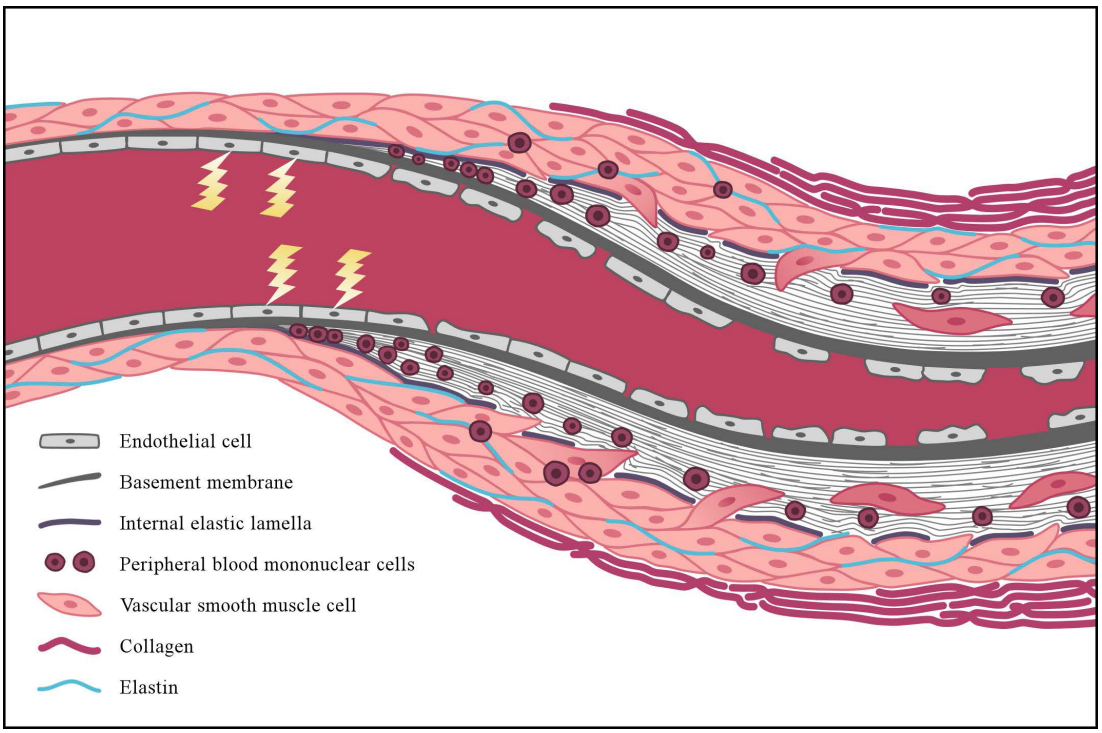

FIGURE 4 | Simplified representation of vascular wall remodeling, leading to critical luminal narrowing of a small vessel as can be seen in patients with SSc. The early events in the pathogenesis of SSc are characterized by endothelial cell (EC) injury, and the lightning bolt represents the initiating factors causing this injury. Reactive oxygen species may play an important initiating role in this process. Following EC injury, EC apoptosis and loss of integrity of the endothelial lining may consequently occur. With the progression of the disease, basement membrane (**continuous gray bold line**) thickening and disruption of the internal elastic lamella are also observed (**dashed line**). Concomitantly, peripheral-blood mononuclear cells (**round nuclear cells**) and vascular smooth muscle cells (VSMCs) (**pink oval-shaped cells**) infiltrate the intimal layer. This process initiates the formation of intimal fibrosis through differentiation of the VSMCs into myofibroblasts, which produce an increased amount of extracellular matrix proteins. Alongside this process, adventitial fibrosis is formed due to increased production of densely packed collagen bundles (**pink bold lines**).

isoprostanes, and circulating total free thiols. Free thiol groups (i.e., −SH) can be oxidized by ROS and other reactive species and are active components of the antioxidant buffer capacity, indicating that their extracellular level can be interpreted as a direct reflection of the overall redox status (Banne et al., 2003; Turell et al., 2013; Cortese-Krott et al., 2017). Lau et al. (1992b) were the first to observe that plasma thiol concentration was reduced in patients with SSc. More recently, we have demonstrated that patients with SSc show decreased levels of free thiols, indicating high oxidative stress. Interestingly, levels of free thiols clearly increased following a cooling experiment of the lower forearm, simulating a Raynaud's attack (van Roon et al., 2017). Following their initial observation, Lau et al. (1992a) also reported increased levels of MDA, which is a measure of lipid peroxidation. Additionally, increased urinary levels of 8-isoprostanes were previously found in SSc patients in the early stages of the disease (Cracowski et al., 2001). It has also been reported that 8-isoprostane in exhaled breath condensate of SSc patients is increased (Tufvesson et al., 2010). Similarly, it was demonstrated that bronchoalveolar lavage fuid from SSc patients with fibrosing alveolitis contained increased levels of 8-isoprostanes (Montuschi et al., 1998). Furthermore, another study showed that the level of 8-isoprostane not only correlates with the severity of pulmonary fibrosis, but also with the extent of renal vascular damage, and immunological abnormalities in SSc (Ogawa et al., 2006). Collectively, these studies have generated increasing interest in the hypothesis that oxidative stress may play a significant role in the pathogenesis of SSc, and subsequently promote vascular injury. However, in order to optimally use these biomarkers, larger studies investigating the value of these markers as predictors of disease (and disease progression) should be performed.

Interventional Strategies

Clear evidence indicates the crucial role played by oxidative stress in the occurrence of SSc vasculopathy. For instance, it has been hypothesized that in SSc, ECs are unable to endure prolonged oxidative stress, either through a lack of antioxidant defense mechanisms or because these antioxidant mechanisms are compromised (Herrick and Matucci Cerinic, 2001). As extensively described elsewhere (Herrick and Matucci Cerinic, 2001; Grygiel-Górniak and Puszczewicz, 2014) several studies have investigated the effect of antioxidants in SSc patients. These studies all shared the postulation that antioxidant supplements would decrease susceptibility to oxidative stress-induced tissue

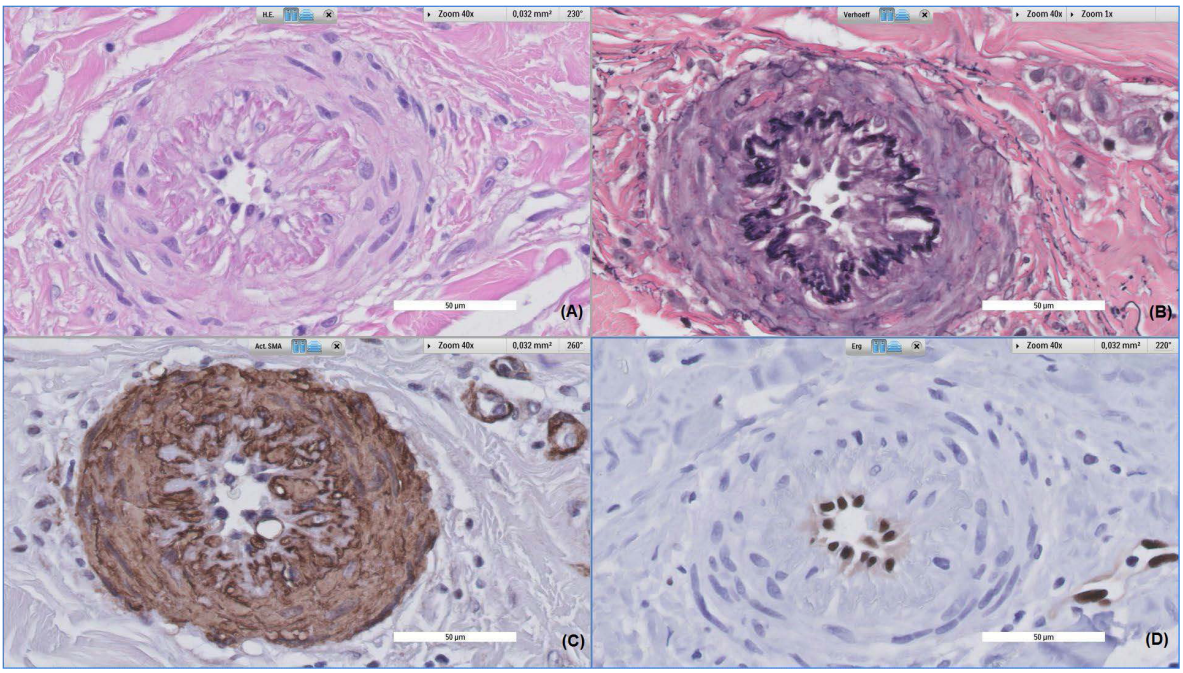

FIGURE 5 | Histopathological alterations of a dermal vessel. Patient, a 62-year-old female, was referred to the dermatologist due to a rash of the lower limbs. All images were taken at 40x magnification, **(A)** shows Hematoxylin and eosin stained tissue, **(B)** shows Verhoeff stained tissue, **(C)** shows alpha-smooth muscle actin stained tissue and **(D)** shows ERG stained tissue. The vessel displayed above shows prominent fibroproliferative alterations, leading to intima and media proliferation; subsequently causing severe narrowing of the vessel lumen and thickening of vessel walls **(A)**. Also, disruption of the internal elastic lamella can be observed **(B)**, with media cells invading the intimal layer **(C)**. This process is accompanied by slight perivascular infiltration of inflammatory cells.

damage. Despite some promising results, larger trials have been disappointing (Simonini et al., 2000; Herrick and Matucci Cerinic, 2001). For instance, Niwa et al. (1985) reported that three out of three SSc patients benefitted from liposomal-encapsulated SOD injections. However, Correa et al. (2014) demonstrated that, when taken orally (1800 mg/day), free radical scavenger *N*-acetyl-L-cysteine (NAC) showed no vasodilator effect on the microcirculation of the hands after 4 weeks of treatment in SSc patients. Conversely, others have reported on the beneficial effect of NAC in SSc patients. For example, NAC has been found to diminish cellular ROS in fibroblast and replenish free cellular thiols (Sambo et al., 2001a,b; Servettaz et al., 2007). In addition, an *in vitro* study, conducted in SSc patients, showed that NAC inhibits fibroblast proliferation and collagen synthesis and reduces the formation of peroxynitrite ($ONOO^-$) by activated lung macrophages (Ammendola et al., 2002). Moreover, Rosato et al. (2009) demonstrated that treatment with NAC led to a reduction in the number of digital ulcers and decreased the number of RP attacks.

Denton et al. (1999) conducted a randomized parallel group study in 40 RP patients (20 SSc patients, 15 primary RP patients, and 5 secondary RP patients). These patients were randomized to receive either 500 mg daily of the lipid-lowering antioxidant probucol or 20 mg daily of nifedipine. Those patients treated with probucol were found to have a significant reduction in the frequency and severity of Raynaud's attacks, as well as a rise in LDL oxidation lag time. In addition, hydrogen sulfide (H_2S), known for its strong antioxidant and vasodilator capacity, was demonstrated to have beneficial effects on bleomycin-induced pulmonary fibrosis in a mice model (Cao et al., 2014). Moreover, Wang et al. (2016) demonstrtaed that H_2S could improve SSc-related organ fibrosis through the inhibition of the inflammatory reaction and the reduction of TGF-β1 expression. These data place H_2S in the spotlight as an effective therapeutic agent in SSc patients with organ involvement. Furthermore, there is some evidence indicating that soluble guanylate cyclase (sGC) may be beneficial in SSc. For instance, large randomized controlled clinical trials conducted with riociguat, which directly stimulates sGC in an NO-independent manner, have shown an increase in mean 6-min walking distance and an improvement of pulmonary vascular resistance and cardiac index (Humbert et al., 2017).

Regarding more conventional therapeutic strategies in SSc, the exploratory study conducted by Erre et al. (2008) suggested that a standard course of iloprost therapy, a potent vasodilator, may reduce oxidative stress in SSc patients. This effect appeared to be more consistent in patients in the early phase of the disease. Furthermore, an experimental study conducted by Rafikova et al. (2013) showed that the ET receptor antagonist bosentan (250 mg/kg/day; days 10–21), which is generally prescribed to reduce the number of new DU, not only prevented an increase in right ventricle peak systolic pressure and right ventricle hypertrophy, but also reduced oxidative stress and protein nitration.

FUTURE PERSPECTIVES

Following Murrell's hypothesis (Murrell, 1993) several studies have investigated the role played by oxidative stress in the pathogenesis of SSc vasculopathy. These studies have not only substantially improved our understanding of this complex disease, but several findings, as presented in this review, support the hypothesis that oxidative stress contributes to, and may even initiate, the occurrence of vasculopathy. This contrasts with the finding that most therapeutic intervention studies with antioxidants have been disappointing. However, some have proposed that these non-conventional therapeutic strategies may only be beneficial in the early stages of the disease, when less severe damage and perhaps lower levels of ROS occur. However, this acts as a double-edged sword. Although universally recognized diagnostic criteria for very early diagnosis of the disease (VEDOSS criteria) have been introduced, these criteria include patients who already have developed EC damage, vasculopathy, and specific antibodies. The inability to identify patients with Raynaud's who are at increased risk of developing SSc is the primary challenge. Any therapy initiated later in the course of the disease may only mitigate disease processes and symptoms, without actually modifying the disease course and outcome. We postulate that interventions administered early in the course of the disease, before the occurrence of irreversible fibrosis, may prevent irreversible changes by altering the damaging properties of ROS and thereby changing the disease's course. Although we are encouraging new trials to investigate the effect of antioxidants on SSc, which should include patients in the early stages of the disease, we emphasize that new trials may only be successful when the underlying pathology is fully clarified.

AUTHOR CONTRIBUTIONS

AA, GD, MF, DM, and HvG have analyzed the data from literature and designed the review. AA wrote the first draft of the manuscript. DM, HvG, MF, and GD wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Reactive Oxygen Species and Pulmonary Vasculature During Hypobaric Hypoxia

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An increasing number of people are living or working at high altitudes (hypobaric hypoxia) and therefore suffering several physiological, biochemical, and molecular changes. Pulmonary vasculature is one of the main and first responses to hypoxia. These responses imply hypoxic pulmonary vasoconstriction (HPV), remodeling, and eventually pulmonary hypertension (PH). These events occur according to the type and extension of the exposure. There is also increasing evidence that these changes in the pulmonary vascular bed could be mainly attributed to a homeostatic imbalance as a result of increased levels of reactive oxygen species (ROS). The increase in ROS production during hypobaric hypoxia has been attributed to an enhanced activity and expression of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), though there is some dispute about which subunit is involved. This enzymatic complex may be directly induced by hypoxia-inducible factor-1α (HIF-1α). ROS has been found to be related to several pathways, cells, enzymes, and molecules in hypoxic pulmonary vasculature responses, from HPV to inflammation, and structural changes, such as remodeling and, ultimately, PH. Therefore, we performed a comprehensive review of the current evidence on the role of ROS in the development of pulmonary vasculature changes under hypoxic conditions, with a focus on hypobaric hypoxia. This review provides information supporting the role of oxidative stress (mainly ROS) in the pulmonary vasculature's responses under hypobaric hypoxia and depicting possible future therapeutics or research targets. NADPH oxidase-produced oxidative stress is highlighted as a major source of ROS. Moreover, new molecules, such as asymmetric dimethylarginine, and critical inflammatory cells as fibroblasts, could be also involved. Several controversies remain regarding the role of ROS and the mechanisms involved in hypoxic responses that need to be elucidated.

Keywords: reactive oxygen species, pulmonary hypertension, hypobaric hypoxia, NADPH oxidase, pulmonary vasculature

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INTRODUCTION

There are two main sources or conditions of hypoxia to which humans are exposed: normobaric hypoxia (at sea level) and hypobaric hypoxia (at high altitudes). Exposure to hypobaric hypoxia can be classified as acute, chronic hypoxia (CH) or chronic intermittent hypoxia (CIH) exposure (Richalet et al., 2002). High-altitude or hypobaric hypoxia exposure leads to a reduction in arterial

oxygen saturation due to a drop in the partial pressure of oxygen (PaO_2), triggering several physiological and/or pathological effects, with pulmonary vascular system changes being among the most important effects (Jensen et al., 1992; Moudgil et al., 2005; Brito et al., 2007). These changes are dependent on exposure time and altitude (Scherrer et al., 2013).

In the clinic, the most well-known CIH is obstructive sleep apnea (OSA), with a prevalence of approximately 14% of the general population. In this condition, the hypoxic state is intermittently maintained for brief periods (Dumitrascu et al., 2013). Although less common, a large body of literature is available on hypobaric hypoxia, which results from living at or ascending to a high altitude. Acute exposure typically applies to tourists and recreational climbers, whereas CH exposure applies to people permanently living at high altitude. Both conditions and their related diseases are rather well characterized (León-Velarde et al., 2005). Over 100 million people are estimated to live at high altitude (Niermeyer et al., 1995; Moore, 2001).

A new model of CIH has been described after the development of mine settlements at high altitudes (over 3000 masl), although this model of hypobaric hypoxia is completely different from other types of intermittent hypoxia such as OSA (Richalet et al., 2002). This type of exposure affects workers commuting to work at high altitude for several days and then resting at sea level for the same period over several years (Richalet et al., 2002). This condition is rather new, and few research studies on the subject are available. Despite some similarities, the changes and mechanisms involved may not be applicable to all types of intermittent hypoxia.

The first vasculature pulmonary phenomenon is hypoxic pulmonary vasoconstriction (HPV) in response to alveolar oxygen pressure. This intrinsic mechanism in the lungs optimizes systemic oxygen delivery by matching perfusion to ventilation (Von Euler and Liljestrand, 1946; Desireddi et al., 2010; Dunham-Snary et al., 2017). This vasoconstrictor effect is modulated by vasoactive substances present in the blood or released from the endothelium and lung parenchyma and can vary with age and species (Leblanc et al., 2013). In contrast, in the systemic vasculature, hypoxia causes a vasodilator effect through the ATP-dependent potassium channel, leading to the relaxation of smooth muscle cells (SMCs) (Weir and Archer, 1995). When alveolar hypoxia is sustained over time, as in CH or in patients with chronic lung disease, HPV can contribute to initiating vascular remodeling and the subsequent development of pulmonary hypertension (PH) and, ultimately, heart failure (Peñaloza et al., 1971; Xu and Jing, 2009; León-Velarde et al., 2010; Rimoldi et al., 2012).

The pathological mechanism of HPV-induced PH involves a wide array of mechanisms and pathways. There is growing evidence that reactive oxygen species (ROS) increase as a result of hypoxia exposure and could play a key role in the pulmonary vasculature responses to hypoxia (Guzy and Schumacker, 2006). ROS can impair the activity of vasodilators or their production and can also elicit inflammatory processes leading to a local inflammatory environment in the pulmonary vasculature (Aggarwal et al., 2013; Wong et al., 2013). Moreover, the molecular mechanism in the pulmonary vasculature that is involved in the response to hypoxia depends on the time and type of exposure and on genetic and individual variabilities (Beall, 2006; Simonson et al., 2010; Scherrer et al., 2013).

For these reasons, this review attempts to provide comprehensive insight into the main factors, sources and mechanisms in the interactions between ROS and other cellular components in the development of vascular responses or diseases due to hypoxia and/or other similar stressors, with an emphasis on pulmonary vasculature under hypobaric hypoxic conditions.

HYPOXIC PULMONARY VASOCONSTRICTION, REMODELING, AND ROS

As mentioned above, HPV is the main and unique response to hypoxia exposure, whose core mechanism resides in calcium flux. Interestingly, in turn, HPV is closely intertwined with ROS activity to elicit this critical response. Therefore, Ca^{2+} plays a fundamental role in PA vasoconstriction (Dunham-Snary et al., 2017). However, whether the internal release of calcium (Gelband and Gelband, 1997) or the influx of extracellular calcium (Weissmann et al., 2006) triggers SMCs contraction is currently debated in the literature.

Thus, it is important to elucidate the role of ROS in the molecular mechanism underlying the increase in the intracellular calcium concentration ($[Ca^{2+}]_i$). Studies have shown that hypoxia-induced increases in $[Ca^{2+}]_i$ in pulmonary artery SMCs (PASMCs) require an increase in ROS signaling, specifically those involving super oxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Jefferson et al., 2004). Thus, oxidative signaling via ROS potentially triggers a Ca^{2+} release from the sarcoplasmic reticulum (SR), allowing extracellular calcium to enter through voltage-dependent calcium channels (VDCCs) and allowing capacitive calcium entry through store-operated calcium channels, thereby resulting in contraction (Wang et al., 2004). Notwithstanding, the main source of calcium in this process remains unclear (Staiculescu et al., 2014). However, studies have shown that extracellular calcium influx caused possibly by hypoxia-induced ROS signaling is necessary to elicit the HPV response since removal of extracellular calcium ions abolishes the hypoxia-induced increase in $[Ca^{2+}]_i$ in SMCs (Weir and Archer, 1995; Desireddi et al., 2010).

A sustained HPV could contribute to starting a vascular remodeling and the subsequent development of PH (Peñaloza et al., 1971; León-Velarde et al., 2010; Rimoldi et al., 2012). Under physiological conditions, the thickness of the vascular wall is maintained by a fine balance between cellular proliferation and apoptosis (Pak et al., 2007; Welsh and Peacock, 2013). If this balance is disrupted in favor of decreased apoptosis and increased proliferation, the vascular wall thickens and can eventually obstruct the vessel lumen. This structural process, which leads to augmented resistance, is known as vascular remodeling (Kato and Staub, 1966). This remodeling effect can be rapidly initiated (Jensen et al., 1992; Moudgil et al., 2005; Engelhardt et al., 2014). In addition, HPV has been documented in several animal models,

including fetal lambs, newborn piglets, rats, and mice (Wong et al., 2013).

This imbalance, which might be triggered by oxidative stress in favor of proliferation, occurs in PAs via different processes: an inhibition of antimitogenic factors (e.g., NO and prostacyclin) and increased synthesis and release of different mitogenic stimuli [endothelin-1 (ET-1), 5-hydroxytryptamine platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)]. There is also a third relevant mechanism: an increased production of extracellular matrix components (Stenmark and Mecham, 1997) through the release of inflammatory mediators [interleukin (IL)-6, IL-8, IL-22, signal transducer and activator of transcription 3 (STAT3), nuclear factor of activated T-cells, stromal cell-derived factor-1, monocyte chemoattractant factor-1 (Kishimoto et al., 2015) and inducible-nitric oxide synthase (iNOS) (Visser et al., 2010; Bansal et al., 2013), among others]. It is noteworthy that not all inflammatory mediators participate in remodeling or PH such as IL-4/IL-13, STAT6, and TLR-MyD88 (El Kasmi et al., 2014).

Controversial Effects of HPV and ROS on Potassium Channels (K^+ Channels)

Although redox signaling has been suggested to be a crucial event in HPV and remodeling, whether an increase or decrease in ROS triggers these processes has been debated (Waypa et al., 2002). First, studies have noted that hypoxia decreases the generation of ROS in PASMCs and shifts the cytosol into a more reduced state, which alters the redox-sensitive thiol groups in the regulatory subunits of Kv channels (Kv2.1), causing the channels to close and altering the resting membrane potential (E_m) in PASMCs. Membrane depolarization then leads to voltage-gated (L-type) calcium channel opening followed by an influx of extracellular calcium into the cytoplasm, resulting in vasoconstriction (Weir and Archer, 1995; Archer et al., 1998). These results are derived from studies on isolated PASMCs, thus simulating a hypoxic environment with powerful reducing agents, such as sodium dithionite, and the subsequent response in potassium currents (hypoxic inhibition of potassium current) may be specific for PASMCs rather than systemic artery SMCs. However, the same studies have shown that the use of sodium dithionite (an oxygen tension-reducing agent) increases the production of superoxide anions and hydrogen peroxide as much as it reduces oxygen tension (Dunham-Snary et al., 2017). Although HPV is an intrinsic response of pulmonary vasculature when there is a K^+ channel blockage, both vasculatures respond with a vasoconstriction (Post et al., 1992). Thus, the most likely explanation of the heterogenic response in systemic and pulmonary vasculature, under hypoxia, would lie in the different cellular mechanisms induced by hypoxia itself (Dunham-Snary et al., 2017). The mentioned discrepancies regarding whether hypoxia leads to an oxidized state with high ROS levels or a reduced state with low ROS levels may be explained by the diversity of methodology or models, including type of artery, severity and type of hypoxia, time of exposure, pH and PCO_2 disturbances, and type of cellular layer.

OXIDATIVE STRESS, ROS, AND HYPOXIA

Oxidative stress is a condition that occurs when there is an increase in ROS at the cellular level. ROS represent a set of reactive species that are derived from oxygen that have one or more unpaired electrons in their outer orbital layer, which make them very unstable and highly reactive. For these reasons, ROS can bind to and oxidize different cellular compounds (lipid, DNA, protein, and cellular membrane, among others), thereby modifying their structure and function and activating cellular signaling (Jefferson et al., 2004; Montezano and Touyz, 2012). Known free radicals include superoxide anion ($O_2^{\bullet -}$), hydroxyl ion (OH), peroxyl (RO_2), and alkoxy agents ($RO^{\cdot}$). Certain non-radicals that are either oxidizing or easily converted into oxidative elements include hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). Notably, there are other oxidative ions that contain nitrogen and can contribute with a nitrosative stress under the name of reactive nitrogen species. The most representative species is peroxynitrite ion ($ONOO^-$), which is derived from NO and $O_2^{\bullet -}$ binding (Radi et al., 1991; Beckman and Koppenol, 1996).

Despite the apparent paradox whereby, in a hypoxic environment, there is more oxidative stress, which has generated a long debate, it is currently agreed that hypoxia and consequently PH are associated with a high level of oxidative stress (Bowers et al., 2004; Guzy and Schumacker, 2006). Several studies have provided evidence of this phenomenon. Using F_2-isoprostane levels in urine as a sensitive biomarker of oxidative stress (Dorjgochoo et al., 2012), ROS levels were observed to be two- to threefold higher in PH patients than in control subjects (Wong et al., 2013). Likewise, higher plasma concentrations of malondialdehyde (MDA), a compound derived from oxidative stress-induced lipid oxidation, have been reported in patients with idiopathic pulmonary arterial hypertension (Irodova et al., 2002) and in rats under hypobaric hypoxia (Lüneburg et al., 2016). Similarly, in endothelial cells, increased levels of 8-hydroxy-deoxyguanosine (8-OHdG), a marker of deoxyribonucleic acid oxidation, have also been found (Bowers et al., 2004; Kishimoto et al., 2015). Thus, an increase in ROS might lead to inflammation, hypertrophy, cellular proliferation, apoptosis, migration, and fibrosis (Wong et al., 2017) while also generating an endothelial dysfunction (Montezano and Touyz, 2012), all of which are components of the remodeling process, which will ultimately manifest in a PH. Therefore, ROS imbalance as a result of hypoxia may be strongly involved in the hypoxic response of pulmonary vasculature at any hypoxic condition, and restoring this balance has prompted several studies.

Main Sources of ROS in the Vascular System Under Hypoxic Conditions

Reactive oxygen species can be produced as a sub-product of the electron transporter chain (ETC) in the mitochondria. The ETC is composed of four mega-complexes that mediate the transfer of electrons down a redox potential gradient through a series of carriers, resulting in the acceptance of

an electron by O_2 and producing ATP and water (Moudgil et al., 2005). A small percentage of total electron flux involves unpaired electrons, resulting in the generation of ROS, notably superoxide radicals, within the mitochondrion. To remove this latter ROS, mitochondria express a unique, inducible isoform of superoxide dismutase (SOD2, manganese SOD or MnSOD). MnSOD transforms toxic superoxide radicals into H_2O_2, which can in turn serve as a diffusible redox mediator in HPV (Archer et al., 1993). Interestingly, the release of superoxides through enzymes and anion channels within the mitochondria of PASMCs can be modulated by diffusible H_2O_2 (Moudgil et al., 2005). Additionally, the relationship between mitochondrial ROS and PA remodeling in response to CH is supported by the observation that depletion of Rieske iron-sulfur protein (RISP), a mitochondrial complex III protein that is required for ROS generation, in mouse ECs and SMCs prevents CH-induced PH (Guzy and Schumacker, 2006; Waypa et al., 2016).

Increasing evidence suggests that the principal source of ROS in the cardiovascular system is the enzymatic complex nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (Moudgil et al., 2005). The NADPH oxidase complex is structurally composed of transmembrane and cytosolic proteins that use NADPH as the electron donor to reduce molecular oxygen to $O_2^{\bullet-}$ and H_2O_2. Studies have characterized seven members of the NOX family of NADPH oxidases (NOX1–5 and dual oxidase 1 and 2), which are expressed within specific tissues and organs (Bache and Chen, 2014), including neurons, skeletal muscle, myocytes, hepatocytes, ECs, hematopoietic cells, stem cells, and cardiomyocytes (Yu et al., 2016). However, NOX4 has been reported as the most prevalent subunit in the cardiovascular system (Chen et al., 2012).

The oxidase role of NADPH has been further supported using animal models with antioxidant overexpression such as catalase or glutathione peroxidase-1 (GP_{x1}). Further, experimental pharmacologic intervention with antioxidants and anti-inflammatory products (probucol, *N*-acetylcysteine, tempol, erdosteine, allicin, pyrrolidine dithiocarbamate, superoxide dismutase, allopurinol, sulfur dioxide, resveratrol, hydrogen water, and EUK-134) have been demonstrated to ameliorate or inhibit PH and right ventricular dysfunction (Waypa et al., 2002; Wong et al., 2013; Kishimoto et al., 2015). However, it is important to highlight that clinical studies have shown inconclusive results using pharmacological or dietary antioxidant treatments, despite the ample evidence on the importance of ROS in experimental models. It is likely that the precise mechanism of ROS and the pharmacokinetics of antioxidants in humans are still not well understood in hypoxic diseases (Wong et al., 2013).

The Oxidase Role of NADPH

There is evidence of increased mRNA and protein expression of a specific NADPH isoform, NOX4, in the lungs of rats exposed to chronic hypobaric hypoxia, presenting PH, in which the concentration of MDA is increased by up to twofold after CIH or CH exposure (Lüneburg et al., 2016). In agreement with this evidence, silencing of NOX4 and p47phox (another subunit of NADPH oxidase) has been shown to attenuate oxidative stress and human and rat PASMC proliferation (Mittal et al., 2012). In addition, both chronic and intermittent hypoxia leads to a notable increase in superoxide radicals, which can impair and decrease NO bioavailability (Barman et al., 2014; Siques et al., 2014).

In contrast, recent studies have shown that NOX4 does not influence the development of hypoxia-induced pathologies in the lung such as HPV or PH (Veith et al., 2016). Supporting the above findings, augmented superoxide anion ($O_2^{\bullet-}$) production has been detected in mice with NOX2- and NOX1-overexpressing SMCs. NOX1- and NOX2-derived $O_2^{\bullet-}$ may contribute to the formation of $ONOO^-$ and subsequently to vascular dysfunction, while NOX4 overexpression increases H_2O_2 formation (Martyn et al., 2006; Serrander et al., 2007; Veith et al., 2016). The reason for the latter resides in the fact that NOX4 may be incapable of scavenging NO in the PA due to a rapid dismutation of $O_2^{\bullet-}$ to H_2O_2 before its release from the enzyme (Takac et al., 2011). Nevertheless, NOX4-produced H_2O_2 can also elicit different effects depending on its concentration, ranging from a beneficial effect on endothelial cell activity in PAs at low H_2O_2 concentrations to the development of SMC hypertrophy at high H_2O_2 concentrations (Schröder et al., 2012; Veith et al., 2016). Therefore, the precise role of each of the NADPH subunits in hypoxia appears to be controversial. However, until recently, NOX4 was considered to be the most relevant according to several authors (Sturrock et al., 2006; Mittal et al., 2007; Nisbet et al., 2009). Drawing a definite conclusion will require further research.

PULMONARY ARTERIAL LAYERS, FIBROBLASTS, AND ROS IN HYPOXIA

Adventitial Cells

Hypoxia induces a more than twofold increase in adventitial thickness in PAs via both fibroblast hypertrophy and hyperplasia and an increase in the surrounding collagen matrix (Meyrick and Reid, 1979). A recent study in rats under CIH showed increased the cellularity of inflammatory cells without changes in lumen size as distinctive features of this condition (Brito et al., 2015). The main trigger or regulator of these processes would be ROS (Welsh et al., 2001). Under hypoxic conditions, fibroblasts appear as key cells as a result of their capacity to migrate into the medial layer and transform into SMCs (Stenmark et al., 2002), thus contributing to vascular remodeling. Moreover, resident adventitial cells could also be activated and reprogrammed for several different behaviors, participating in remodeling and SMC tone (Welsh and Peacock, 2013).

Thus, under hypoxic conditions, adventitial fibroblasts would also act via the generation of matrix proteins (Sartore et al., 2001), which contribute to the narrowing of the vascular lumen, via cytokines, growth factors, and inflammatory mediators [IL-6, STAT3, hypoxia-inducible factor-1 (HIF-1), and C/EBPβ]. The last one has been shown to be paracrine regulators of vascular macrophages for chronic inflammation, remodeling, and PH (El Kasmi et al., 2014).

The molecular mechanism by which hypoxia stimulates proliferation in PA fibroblasts is under study. Nevertheless,

there is evidence that mitogen-activated protein (MAP) kinase, Erk1/2 and related stress-activated kinases, such as Jnk and p38 MAP kinase, are key regulators of cell proliferation and can be activated in response to hypoxic stress (Seko et al., 1996; Robinson and Cobb, 1997; Das et al., 2001). Further support for the role of hypoxia-induced oxidative stress in the activation of these protein kinases has been described (Dworakowski et al., 2006; Schröder et al., 2009; Montezano and Touyz, 2012; Yamada et al., 2012). In addition, recent studies show that HIF-1α stabilization in all PA cells elicits the production of ROS via p22phox-containing NADPH oxidase. Therefore, HIF-1α-induced ROS production might contribute to the activation of PA fibroblasts and could be another mechanism by which hypoxia induces PA fibroblast proliferation (Welsh et al., 2001). Controversially, a recent cell culture study reported that ROS levels in human fibroblasts decrease under hypoxic conditions (Sgarbi et al., 2017); however, this finding requires further verification.

Another mechanism for fibroblast proliferation under hypoxia would involve their capacity to transdifferentiate into other cell types such as myofibroblasts through a complex network of microenvironmental factors and extracellular matrix components (Stenmark et al., 2002).

Smooth Muscle Cells

It is well known that medial thickness greatly determines pulmonary vascular resistance. The pre-capillary segment of the pulmonary vascular bed contributes to pulmonary vascular resistance and determines PA pressure. Under hypoxic conditions, these vessels enhance their muscularization (Meyrick and Reid, 1979), representing a key feature of hypoxic pulmonary vascular remodeling (Pak et al., 2007). This effect has been observed in CH (Penaloza and Arias-Stella, 2007) and CIH, but to a lesser extent (Brito et al., 2015). Whether acute hypoxia has proliferative effects on PASMCs is controversial, though several studies have demonstrated either increased ROS levels or cellular proliferation in acute hypoxia (Wedgwood et al., 2001).

Vascular SMCs play important roles in the physiological regulation of vascular tone and vascular remodeling. The mechanisms by which hypoxia lead pulmonary vessels to constrict and coronary vessels to relax are subject to ongoing research. Hypoxia-induced closure of Kv channels could be the result of a change in cytoplasmic ROS or redox status in PASMCs. Moreover, NADPH oxidase has been shown to bind the β-subunit of Kv channels, thus supporting a link between ROS and K^+ channels and cellular excitability (Wu et al., 2007).

Notably, ET-1 is upregulated during hypoxia and has been demonstrated to influence SMC proliferation via NADPH oxidase-derived superoxides and to contribute to the pathogenesis of hypoxic-induced PH (Madden et al., 1992; Li et al., 1994; Liu et al., 2007). Moreover, administration of ET-1 significantly increases ROS production in both PASMCs and coronary artery SMCs (Wu et al., 2007), whereas inhibition of NADPH oxidase in bovine fetal PASMCs activates the apoptosis process and avoids ET-1-mediated SMC proliferation (Wu et al., 2007).

The role of ROS SMC proliferation is also a controversial issue. While one study has shown that acute hypoxia significantly reduces superoxide production in both human PASMCs and coronary arterial SMCs (Gupte and Wolin, 2006), others have demonstrated increased ROS production in pulmonary arteries due to acute hypoxia and CIH (Wedgwood et al., 2001; Schumacker, 2011; Siques et al., 2014). There are several explanations for the lack of superoxide: a differential regulation of NADPH oxidases due to higher basal cytosolic NADPH oxidase levels in PAs (Gupte and Wolin, 2006), a subcellular localization of NOX, which may be critical for the activation of downstream signaling, and differences in SOD activity between PASMC and coronary artery SMCs. Regrettably, the available literature does not allow a definitive explanation.

Another possible pathway involved in SMC proliferation could be explained by ROS-activated p38MAPK, as demonstrated in hypoxia-induced proliferation and contraction in rat PAs (Karamsetty et al., 2002). Remarkably, this latter effect has not been observed in systemic vasculature (Sigaud et al., 2005). In addition, carbonylation of annexin A1 may contribute to the development of vascular remodeling and PH since ET-1 and serotonin (5-HT) in PASMCs could promote the carbonylation of annexin A1 protein (Wong et al., 2008). Annexin A1 exerts anti-inflammatory, antiproliferative, and proapoptotic effects. Consequently, inhibition of protein carbonylation may be a novel therapeutic target for hypobaric hypoxia PH (Wong et al., 2013).

Endothelial Cells

The EC layer is in a unique position that allows it to respond to circulatory and blood factors, acting as a vasculature integrator, modulator, and signal transducer via paracrine signaling (Furchgott and Zawadzki, 1980).

Under hypoxic conditions, an endothelial dysfunction occurs that not only alters the vascular tone but also contributes with inflammatory and proliferative mediators, ultimately leading to vascular remodeling (Griendling and Ushio-Fukai, 2000). Thus, ECs regulate the vascular tone and pulmonary remodeling by producing vasodilator and antiproliferative NO or vasoconstrictive pro-proliferative factors (ET-1, angiotensin II, thromboxane A_2 and PDGF-B; Pak et al., 2007) that are exclusive to ECs but not to SMCs (Ambalavanan et al., 1999).

A main endogenous vasodilator and antiproliferative is NO, which is synthetized in ECs by endothelial nitric oxide synthase (eNOS), through stimulation of soluble guanylyl cyclase (sGC) to increase the level of cyclic GMP in SMCs (Förstermann et al., 1986). Therefore, lesser NO availability would lead to endothelial dysfunction (Cai and Harrison, 2000; Wolin et al., 2010; Frazziano et al., 2012). Increased ROS levels under hypoxia have been widely demonstrated during this review and would play a key role in impairing NO production and bioavailability (Cai and Harrison, 2000; Siques et al., 2014). The described mechanisms through which ROS would alter NO levels in ECs include NO degradation, $ONOO^-$ production, and increasing oxidative stress (Radi et al., 1991; Millatt et al., 2003; Sun et al.,

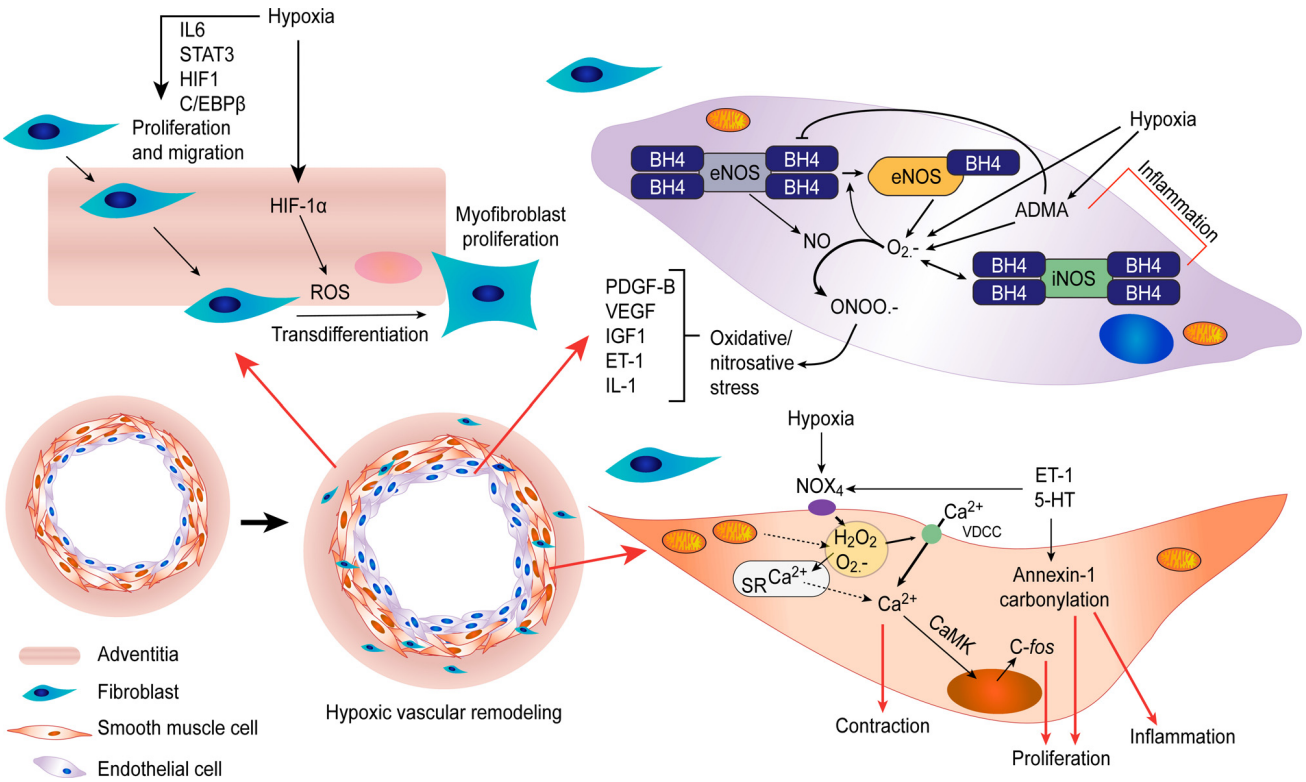

FIGURE 1 | Proposed scheme: the most feasible and hypothetic mechanisms involved in the relationship between ROS and pulmonary vascular cells under hypobaric hypoxic conditions are summarized. Ca^{2+}, calcium; ROS, reactive oxygen species; SR, sarcoplasmic reticulum; 5-HT, serotonin; ET-1, endothelin-1; IL-6, interleukin 6; IL-1, interleukin 1; STAT3, signal transducer and activator of transcription 3; HIF-1, hypoxia-inducible factor 1; C/EBPβ, CCAAT/enhancer-binding protein beta; PDGF-B, platelet-derived growth factor subunit B; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor; $ONOO^-$, peroxynitrite; ADMA, asymmetric dimethylarginine; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; BH4, tetrahydrobiopterin; VDCCs, voltage-dependent calcium channels; $O_2^{\bullet-}$, superoxide anion; H_2O_2, hydrogen peroxide; CaMK, Ca^{2+}/calmodulin-dependent protein kinase.

2010; Dumitrascu et al., 2013), stimulation of uncoupled eNOS formation (BH4 decreased) (Sun et al., 2010), stimulation of iNOS activity and enhancement of O_2-production (Loscalzo, 2001).

Interestingly, enhanced associations between iNOS and asymmetric dimethylarginine (ADMA) level leading to airway inflammation and also in the vasculature have been described (Wells and Holian, 2007; Visser et al., 2010). ADMA induces a decrease in endothelium-dependent vasodilation through the inhibition of eNOS (Böger, 2006; Wilcken et al., 2007). ADMA also interacts with ROS in the vasculature by disturbing the usual flow of electrons between the two domains of eNOS, becoming a generator of superoxide radicals instead of NO (Sydow and Münzel, 2003). Recently, it has been demonstrated that exposure to CIH and CH induces a steady increase of ADMA in human (Lüneburg et al., 2017) and rat models (Lüneburg et al., 2016). Therefore, this mechanism could also contribute to the hypoxic inflammatory status, hypoxic endothelial dysfunction, and subsequent vascular remodeling.

A limitation of this review is that it focuses mainly on hypobaric hypoxia, which is the hypoxia experienced at high altitude, but it was also necessary to include studies conducted in either simulated hypobaric or normobaric hypoxia. This could introduce some bias because it has been well determined that different responses can occur according to the source of the hypoxia (Savourey et al., 2003; Bonetti et al., 2009; Debevec and Millet, 2014).

CONCLUSION

This review provides information supporting the role of oxidative stress (mainly ROS) in the pulmonary vasculature's responses under hypobaric hypoxia and depicting possible future therapeutic and research targets. NADPH oxidase-produced oxidative stress is highlighted as a major source of ROS. Moreover, new molecules, such as ADMA, and critical inflammatory cells as fibroblasts, could be also involved. Several controversies still remain regarding the roles of ROS and the mechanisms involved in hypoxic responses. Despite the latter, these mechanisms, independently or cooperatively, could contribute to the responses of the pulmonary vasculature to hypoxia, ultimately resulting in PH.

Finally, a schematic summarizing the most feasible and hypothetic mechanisms involved in the relationship between

ROS and pulmonary vascular cells under hypobaric hypoxic conditions, as a result of this review, is provided in **Figure 1**.

AUTHOR CONTRIBUTIONS

PS, JB, and EP equally contributed to the design of the review, gathering of information, analysis of literature, and drafting of the manuscript. All of the authors approved the final manuscript and agreed to be accountable for all aspects of the work.

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Long-Term Consumption of Cuban Policosanol Lowers Central and Brachial Blood Pressure and Improves Lipid Profile With Enhancement of Lipoprotein Properties in Healthy Korean Participants

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Kim S-J, Yadav D, Park H-J, Kim J-R and Cho K-H (2018) Long-Term Consumption of Cuban Policosanol Lowers Central and Brachial Blood Pressure and Improves Lipid Profile With Enhancement of Lipoprotein Properties in Healthy Korean Participants. Front. Physiol. 9:412. doi: 10.3389/fphys.2018.00412

Metabolic syndrome is closely associated with higher risk of hypertension, cardiovascular disease (CVD), diabetes and stroke. The aim of the present study was to investigate the long-term effects of policosanol supplementation on blood pressure (BP) and the lipid profile in healthy Korean participants with pre-hypertension (systolic 120–139 mmHg, diastolic 85–89 mmHg). This randomized, double-blinded, and placebo-controlled trial included 84 healthy participants who were randomly assigned to three groups receiving 10 mg of policosanol, 20 mg of policosanol, or placebo for 24 weeks. The BP, lipid profile, and anthropometric factors were measured pre- and post-intervention and then compared. Based on an average of three measurements of brachial BP, the policosanol 20 mg group showed the most significant reduction in average systolic BP (SBP) from 138 ± 12 mmHg at week 0 to 126 ± 13 mmHg at week 24 ($p < 0.0001$). The policosanol 20 mg group also showed significant reductions in aortic SBP and DBP up to 9% ($p = 0.00057$) and 8% ($p = 0.004$), respectively compared with week 0. Additionally, blood renin and aldosterone levels were significantly reduced in the policosanol 20 mg group up to 63% ($p < 0.01$) and 42% ($p < 0.05$), respectively, at week 24. For the blood lipid profile, the policosanol 10 mg and 20 mg groups showed significant reductions in total cholesterol (TC) of around 8% ($p = 0.029$) and 13% ($p = 0.0004$), respectively, at week 24 compared with week 0. Serum HDL-C level significantly increased up to 16% and 12% in the policosanol 10 mg ($p = 0.002$) and 20 mg ($p = 0.035$) group, respectively. The study results suggest that long-term policosanol consumption simultaneously reduces peripheral BP as well as aortic BP accompanied by elevation of HDL-C and % HDL-C in TC in a dose-dependent manner.

Keywords: policosanol, blood pressure, lipoproteins, apolipoprotein A-I, glycation

INTRODUCTION

Hypertension is a major risk factor for the development of cardiovascular disease (CVD), which is often accompanied by other risk factors such as dyslipidemia (Nelson, 2013; Lee et al., 2017). It is well-known that serum high-density lipoprotein (HDL-C) is inversely correlated with incidence of aging-related diseases such as CVD, diabetes, and Alzheimer's disease (McGrowder et al., 2010; Reitz et al., 2010; Hirano et al., 2014). The HDL-C/total cholesterol (TC) ratio (%), rather than HDL-C (mg/dL), is more important in predicting the risk of incident hypertension (Halperin et al., 2006).

In addition to HDL-C quantity, it has been firmly established that HDL quality and functionality are more important in the suppression of aging-related diseases (Eren et al., 2012). There are a limited number of approaches to increasing HDL-C quantity comprising dietary foods or drugs to elevate the concentration of HDL-C and enhance HDL functionality (Sahebkar et al., 2016). Cuban policosanol (PCO) was reported to lower TC and low-density lipoprotein cholesterol (LDL-C) as well as increase HDL-C levels by inhibiting cholesterol synthesis and increasing LDL-C excretion (Janikula, 2002; Lee et al., 2016; Kim et al., 2017).

Policosanol potentiates the beneficial functions of HDL as well as its antioxidant, anti-glycation, and anti-atherosclerotic activities (Lee et al., 2016; Kim et al., 2017). Although short-term consumption of policosanol has been shown to reduce brachial blood pressure (BP) accompanied by increased HDL-C concentration and antioxidative functionality, correlations of BP reduction with enhancement of HDL-C in TC remain to be investigated in a long-term study.

Quality of HDL is also recognized as important factor in the suppression of atherosclerotic progression. Impaired functionality of HDL such as cholesterol efflux is closely associated with atherosclerotic CVD (Rohatgi et al., 2014).

A previous study reported that short-term consumption of policosanol enhanced HDL-C particle size, and reconstituted HDL (rHDL) containing policosanol increased cholesterol efflux capacity via up-regulation of ABCA1 for excretion (Kim et al., 2017). Although there are inconsistent data related to the efficacy of lowering cholesterol with policosanol therapy (Cubeddu et al., 2006; Francini-Pesenti et al., 2008), Gong et al. (2018) in a recent meta-analysis comprising 22 studies reported that policosanol could be used to lower lipid content and as a safe drug to elevate HDL-C levels (Gong et al., 2018). However, until now, there has been no *in vivo* or human study on the HDL-C/TC ratio and HDL functionality in relation to the central BP-lowering effect of policosanol in human participants over the long-term. In recent years, the prevalence of pre-hypertension is widely increased around the world including Korea (Kim and Lee, 2015). Pre-hypertensive subjects are more risky for causing hypertension and related morbidity. Therefore it is prudent to study the dietary foods or drugs on the pre-hypertensive population to further reduce the actual burden of hypertension and hypertension related mortality in the future. We hypothesized that consumption of policosanol for 24 weeks would reduce both brachial and central aortic BP along with beneficial effects on lipid parameters. Therefore, we tested the physiological effects of policosanol consumption on brachial and central BP, and lipoprotein functionality in healthy Korean subjects.

MATERIALS AND METHODS

Policosanol

Policosanol and placebo tablet were obtained from Rainbow & Nature Pty, Ltd (Thornleigh, NSW, Australia). Policosanol (sugar cane wax alcohol, SCWA) consists of considerable chains of alcohol of various lengths. More than 90% of policosanol contents were higher aliphatic alcohols. Individual alcohols present in policosanol are 1-tetracosanol ($C_{24}H_{49}OH$; molecular weight—MW: 354.7 mμ) $\leq$ 2%; 1-hexacosanol ($C_{26}H_{53}OH$; MW: 382.4 mμ$\leq$ 4.5–10%; 1-heptacosanol ($C_{27}H_{55}OH$; MW: 396.4 mμ) $\leq$ 5%; 1-octacosanol ($C_{28}H_{57}OH$; MW: 410.5 mμ)) $\leq$ 60–70%; 1-nonacosanol ($C_{29}H_{59}OH$; MW: 424.8 mμ) $\leq$ 2%; 1-triacontanol ($C_{30}H_{61}OH$; MW: 438.5 mμ) $\leq$ 10–15%; 1-dotriacontanol: ($C_{32}H_{65}OH$; MW: 466.5 mμ) $\leq$ 3–8%; 1-tetratriacontanol ($C_{34}H_{69}OH$; MW: 494.5 mμ) $\leq$ 2%.

Participants

We recruited healthy male and female volunteers who had prehypertension (systolic 120–139 mmHg, diastolic 80–89 mmHg). All recruited participants were pre-screened for eligibility criteria and the inclusion were as follows: age 18–65 years old who had pre-hypertension without any complaint of endocrinological disorder. Heavy alcohol drinkers (>30 g EtOH/day) and those who consumed drugs related to hyperlipidemia, diabetes mellitus, or hypertension were excluded. All participants had unremarkable medical records without illicit drug use or past history of chronic diseases. On the first visit day, all participants casted dice for randomized grouping for group 1 (placebo), group 2 (policosanol 10 mg), and group 3 (policosanol 20 mg). The selected participants consumed policosanol for 24 weeks, based on the represented study design (**Figure 1**). The study was approved by the Institutional Review Board at Yeungnam University (Gyeongsan, South Korea) endorsed the protocol (IRB #7002016-A-2016-021) and the participants signed an informed consent form prior to research commencement.

Study Design

This study was a double-blinded, randomized, and placebo-controlled trial with a 24-week treatment periods. Participants were directed to take one tablet per day containing policosanol either 10 or 20 mg of sugar cane wax alcohol or placebo consisting of a dextrin and lactose mixture, manufactured by CosmaxBio Inc. (Jecheon, Korea). Other materials used to make the tablets were corn starch, cellulose, gelatin, stearic acid, etc. The ingredients, manufacturing process, and facility were approved by the Korean FDA.

All participants received advice to avoid excess alcohol drinking (less than 30 g of Et-OH per day). They were also instructed to avoid intense exercise (less than 30 min per day at 60–80% maximum capacity). If subjects had a sedentary lifestyle before commencement, we advocated them to balance their

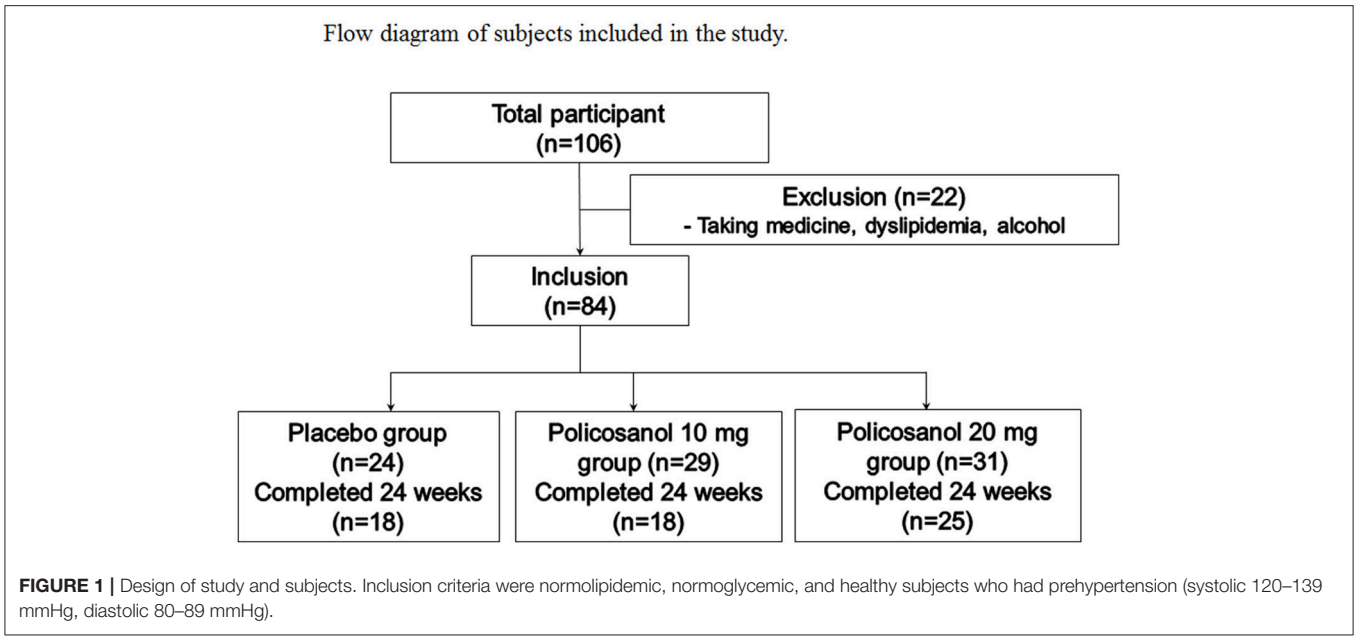

FIGURE 1 | Design of study and subjects. Inclusion criteria were normolipidemic, normoglycemic, and healthy subjects who had prehypertension (systolic 120–139 mmHg, diastolic 80–89 mmHg).

lifestyle during the policosanol consumption period to avoid bias due to excess exercise or other life style habits.

Anthropometric Analysis and Physical Activity Assessments

The participants were measured for different anthropometrical parameters such as height, body weight, body mass index (BMI), subcutaneous fat (kg), and visceral fat mass (kg) individually at the same time of day at 4-week intervals using an X-scan plus II body composition analyzer (Jawon Medical, Gyeongsan, Korea).

Measurement of Blood Pressure

BP was measured each visit for a total of three times, and the average was recorded at 4-week intervals using three BP measuring instruments. We used a digital BP device (Omron HBP-9020, Kyoto, Japan), mercury sphygmomanometer, and the SphygmoCor system (AtCor Medical, Sydney, Australia) to measure peripheral and central aortic BP (Yadav et al., 2018). All manual measurements or procedures were carried out by a licensed technician (S.J.K.).

The idea of using three different techniques was to obtain more accurate results, as a previous report suggested discrepancies in BP measurement between these devices (Li et al., 2013). Instruments such as the Omron digital BP device and mercury sphygmomanometer are commonly used in hospitals to estimate BP. However, in epidemiological studies, focusing on central aortic BP is limited, and therefore we represent all data in this long-term policosanol study. It is known that central aortic BP provides better prognostic information than brachial BP due to close proximity to important organs such as the heart, brain and kidneys. A meta-analysis of 11 longitudinal studies reported that measurement of central aortic BP could have a good clinical significance and be a better predictor of cardiovascular events (Vlachopoulos et al., 2010). Central aortic pressure waveform represents different components such as augmentation pressure and augmentation index. Augmentation pressure is the difference between early aortic systolic pressure and late aortic systolic pressure. Augmentation index is measured based on the ratio of augmentation pressure to central aortic pulse pressure.

Blood Analysis

Blood was obtained from participants after overnight fasting. Blood was collected using a vacutainer (BD Biosciences, Franklin Lakes, NJ, USA) containing EDTA (final concentration of 1 mM) at weeks 0 and 24 by low-speed centrifugation (3,000 g) and stored at −80°C until analysis. To analyze plasma, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and glucose levels were measured using commercially available kits (Cleantech TS-S; Wako Pure Chemical, Osaka, Japan).

Angiotensin-Converting Enzyme and Aldosterone-Renin Analysis

Plasma aldosterone and renin level were measured by radioimmuno assay (RIA) using an instrument (1470-Gamma Counter, PerkinElmer) via the Seegene Medical Foundation (Seoul, Korea). Angiotensin-converting enzyme (ACE) activity was measured by an enzymatic assay kit obtained from Buhlmann Laboratories (Schönenbuch, Switzerland). Homocysteine was measured by chemiluminescent immunoassay using a Liquid stable Reagnet kit obtained from Axis-Shield Diagnostics Ltd. (Luna Place Technology Park, United Kingdom) via the Seegene Medical Foundation (Seoul, Korea).

Characterization of Lipoproteins

Very low-density lipoprotein (VLDL, $d < 1.019$ g/mL), low-density lipoprotein (LDL, $1.019 < d < 1.063$), high-density lipoprotein$_2$ (HDL_2, $1.063 < d < 1.125$), and high-density lipoprotein$_3$ (HDL_3, $1.125 < d < 1.225$) were isolated

from individual plasma samples of each group via sequential ultracentrifugation (Havel et al., 1955), and density was adjusted by addition of NaCl and NaBr in accordance with standard protocols. Samples were centrifuged for 22 h at 10°C and 100,000 g using a Himac CP-100NX (Hitachi, Tokyo, Japan) at the Instrumental Analysis Center of Yeungnam University. To analyze lipoproteins, TC and TG levels were measured using commercially available kits (Cleantech TS-S; Wako Pure Chemical, Osaka, Japan). Protein concentrations of lipoproteins were determined via Lowry protein assay, as modified by Markwell et al. (1978). Expression levels of apoA-I (28 kDa) were determined by SDS-PAGE and immunodetection using apoA-I polyclonal antibody (ab7613) obtained from Abcam (Cambridge, UK).

To assess the degree of lipoprotein oxidation, the concentration of oxidized species in lipoproteins was determined by the thiobarbituric acid reactive substance (TBARS) assay method using malondialdehyde as a standard (Blois, 1958). Extent of glycation between the three groups based on advanced glycation end products (AGEs) in HDL_2 and HDL_3 lipoproteins was determined from fluorometric intensities at 370 nm and 440 nm as described previously (McPherson et al., 1988), using a spectrofluorometer LS55 (Perkin Elmer, Shelton, CT, USA) with the WinLab software package (version 4.0).

LDL Oxidation

LDL was oxidized by incubation of the LDL fraction with $CuSO_4$ (final concentration of 10 μM) for 4 h at 37°C. oxLDL was then filtered through a 0.22-μm filter (Millex; Millipore, Bedford, MA) and analyzed by thiobarbituric acid reactive substances (TBARS) assay to determine the extent of oxidation using a malondialdehyde (MDA) standard (Blois, 1958). To verify spectroscopic data, oxidized samples were subjected to 0.5% agarose gel electrophoresis to compare electromobilities**.** The migration of each lipoprotein was dependent on its intact charge and size. Gels were then dried and the bands stained with 0.125% Coomassie Brilliant Blue.

Data Analysis

All data are expressed as the mean ± SD from at least three independent experiments with duplicate samples. For human data analysis, all data were analyzed by normality test; normally distributed data were compared using Student *t*-test. Correlation analysis was carried out using Pearson's test. $p < 0.05$ was defined as being significant. Statistical analysis was performed using the SPSS software program (version 23.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

Comparison of Body Composition, Brachial BP, and Aortic BP After 24 Weeks of Therapy

Table 1 represents the data on body composition, peripheral BP, and central BP at baseline (week 0) and after 24 weeks. After 24 weeks of policosanol consumption, there were no significant changes in BMI, as measured values among all groups were around 22–24 (kg/m^2). Subcutaneous fat and visceral fat contents were similar among all groups between weeks 0 and 24.

From the SphygmoCor measurements, there was no significant reduction of BP in group 1, whereas, groups 2 and 3 showed significant SBP reductions up to 7% (130 ± 15 mmHg, $p = 0.0335$) and 10% (123 ± 11 mmHg, $p = 0.0003$), respectively. Especially in group 3, brachial SBP and DBP were reduced to normal level around 126 and 79 mmHg, respectively) after 24 weeks of policosanol therapy. Omron digital BP monitor measurement also revealed that group 3 showed normal SBP and DBP with 12% (134 ± 20 mmHg, $p = 0.001$) and 13% reductions (122 ± 10 mmHg, $p = 0.004$), respectively, compared with week 0. Mercury sphygmomanometer measurement revealed that group 3 showed normal range of SBP and DBP at week 24 with 11% reduction (121 ± 9 and 75 ± 9 mmHg, $p = 0.001$) compared with week 0. Similar to the above, groups 1 and 2 did not show any significant change in SBP or DBP.

Based on an average of three measurements, at week 24, group 3 (policosanol 20 mg) showed significantly reduced average SBP and DBP levels up to 10% (126 ± 13 mmHg, $p < 0.0001$) and 9% (79 ± 8 mmHg, $p < 0.0001$), respectively, whereas group 2 showed 6% reductions in SBP (128 ± 6 mmHg, $p = 0.016$) and DBP (83 ± 12 mmHg, $p = 0.014$) compared with week 0.

Central BP measurements showed that aortic SBP and DBP in group 3 were reduced up to 10% (113 ± 12 mmHg, $p = 0.001$) and 8% (84 ± 5 mmHg, $p = 0.004$) at 24 weeks, compared with week 0. Group 2 showed an 8% reduction in SBP (120 ± 13 mmHg, $p = 0.001$) and 7% reduction in DBP (86 ± 8 mmHg, $p = 0.014$) after 24 weeks, whereas group 1 did not any show significant change. Mean arterial pressure at week 24 was reduced in groups 2 and 3 up to 7 mmHg (97 ± 11 mmHg, $p < 0.05$) and 10 mmHg (93 ± 9 mmHg, $p < 0.01$) respectively, whereas group 1 showed no change. These results suggest that policosanol consumption for 24 weeks significantly reduced aortic BP as well as brachial (peripheral) BP in a dose-dependent manner.

Changes in Serum Lipid Profile After 24 Weeks of Therapy

Table 2 indicates the changes of blood profile after 24 weeks of therapy. Regarding blood TC level, groups 2 and 3 showed significant reductions in TC of around 8% (180 ± 8 mg/dL, $p = 0.029$) and 10% (161 ± 7 mg/dL, $p < 0.001$), respectively, whereas blood TG level did not change significantly. Serum HDL-C level did not change in group 1 (around 39 mg/dL) over 24 weeks, whereas group 2 showed a significant increase from 36 ± 2 to 42 ± 3 mg/dL. Group 3 showed the most significant increase in HDL-C level from 39 ± 2 mg/dL at week 0 to 44 ± 2 mg/dL ($p = 0.007$). Group 1 did not show any change in the HDL-C/TC ratio (% HDL-C) between week 0 and 24, and the calculated value was around 22%. Group 3 showed an HDL/TC ratio of 28% ($p = 0.00014$) at week 24 compared to 21% at week 0. Group 3 showed the largest reduction in calculated LDL-C up to 20% (101 ± 30 mg/dL), whereas groups 2 showed 14% reductions, respectively. Blood glucose level was not altered in group 1, whereas groups 2 and 3 showed significant reductions up to 8% (90 ± 11 mg/dL, $p = 0.03$ and 86 ± 12 mg/dL, $p =$

TABLE 1 | Change of blood pressure profile after 24 weeks consumption.

		Group 1 Placebo (n = 24, M14/F10)		Group 2 Policosanol 10 mg (n = 29, M21/F8)		Group 3 Policosanol 20 mg (n = 31, M22/F9)	
Age		32 ± 14		34 ± 16		31 ± 12	
Body composition		**Week 0**	**Week 24**	**Week 0**	**Week 24**	**Week 0**	**Week 24**
BMI		22.3 ± 1.5	22.5 ± 1.4	23.2 ± 1.8	23.4 ± 1.8	23.7 ± 1.5	23.8 ± 1.3
Subcutaneous fat (kg)		12.3 ± 2.3	12.4 ± 2.1	13.6 ± 2.5	14.1 ± 2.7	13.8 ± 2.5	14.4 ± 1.8
Visceral fat (kg)		1.7 ± 0.5	1.6 ± 0.4	2.0 ± 0.5	2.1 ± 0.6	2.0 ± 0.5	2.1 ± 0.4
Peripheral BP (mmHg)		**Week 0**	**Week 24**	**Week 0**	**Week 24**	**Week 0**	**Week 24**
SphygmoCor	Systolic	135 ± 12	132 ± 11	139 ± 16	130 ± 15**	136 ± 8	123 ± 11***
	Diastolic	88 ± 8	84 ± 8	89 ± 10	87 ± 11	87 ± 7	81 ± 9*
Omron	Systolic	134 ± 12	135 ± 10	140 ± 18	134 ± 20*	138 ± 11	122 ± 10***
	Diastolic	84 ± 9	83 ± 9	85 ± 12	84 ± 15	82 ± 10	72 ± 10**
Mercury	Systolic	131 ± 6	129 ± 8	134 ± 12	130 ± 17*	135 ± 6	121 ± 9***
	Diastolic	83 ± 5	81 ± 8	84 ± 10	82 ± 10	84 ± 5	75 ± 9*
Average	Systolic	138 ± 9	132 ± 6	135 ± 13	128 ± 6**	138 ± 12	126 ± 13***
	Diastolic	87 ± 6	83 ± 6	86 ± 10	83 ± 12	87 ± 7	79 ± 8**
Aortic BP (mmHg)		**Week 0**	**Week 24**	**Week 0**	**Week 24**	**Week 0**	**Week 24**
Aortic	Systolic	123 ± 12	117 ± 9	130 ± 14	120 ± 13*	123 ± 13	113 ± 12***
	Diastolic	90 ± 8	85 ± 7	92 ± 10	86 ± 8*	91 ± 7	84 ± 5**
Mean arterial pressure		104 ± 9	100 ± 8	104 ± 11	97 ± 11*	103 ± 7	93 ± 9**
Pulse pressure		32 ± 8	32 ± 4	35 ± 10	30 ± 5	32 ± 5	29 ± 5

*Data are expressed as mean±SD. ***$p < 0.001$ vs. week 0; **$p < 0.01$ vs. week 0; *$p < 0.05$ vs. week 0 in each group. AU, arbitrary unit; BP, blood pressure; BMI, body mass index.*

0.05, respectively). Homocysteine level was also similar between the groups at weeks 0 and 24.

Renin, Aldosterone, and ACE

At week 0, group 3 (**Table 2**) showed the highest renin level while groups 1 and 2 showed similar renin levels. After 24 weeks, blood renin level was significantly reduced in groups 2 and 3 up to 35% and 63%, respectively, whereas group 1 did not show any significant change. Aldosterone levels in all groups were 19–29 ng/dL at week 0, whereas, groups 2 and 3 showed significant reductions up to 35% (19.6 ± 2.1 ng/dL) and 42% (14 ± 1.5 ng/dL) respectively, compared with week 0. ACE activity was unchanged after 24 weeks, and all groups showed similar level of around 30–34 units at weeks 0 and 24.

Correlation Analysis Between Brachial Blood Pressure and Lipid Profiles

We studied correlations between brachial BP and lipid profiles based on Pearson's correlation coefficient. At baseline, we observed correlations among SBP, DBP, and lipid levels based on total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and percentage (%) of HDL-C and TG/HDL-C. Analysis at baseline in the first group revealed that SBP had a positive correlation with DBP and a negative correlation with TG (**Table 3**). A significant inverse correlation was observed between TC and % of HDL-C. After 24 weeks of therapy in group 1, only SBP was significantly correlated with DBP ($p = 0.032$). The study did not find any significance between BP and lipid profiles after 24 weeks of placebo therapy (**Table 3**).

In group 2, SBP was significantly correlated with DBP ($p = 0.0001$) at week 0. None of the other parameters showed statistical significance. However, DBP showed positive correlations with TG and TG/HDL-C levels (**Table 4**). In group 2, correlations among SBP, DBP, and lipid parameters were mildly improved at week 24. SBP was significantly correlated with measurement of DBP ($r = 0.555$, $p = 0.039$), TG level ($r = 0.577$, $p = 0.031$), and the TG/HDL-C ($r = 0.700$, $p = 0.005$) ratio (**Table 4**). Additionally, SBP was negatively correlated with HDL-C ($r = -0.615$, $p = 0.019$) and % of HDL-C ($r = -0.737$, $p = 0.003$). The study did not observe any significant values when correlating DBP with lipid parameters.

In group 3 at baseline, SBP showed positive correlations, with DBP, TG, and TG/HDL-C and a negative correlation with HDL-C level (**Table 5**). DBP showed significant positive correlations with TG and TG/HDL-C.

After 24 weeks of policosanol therapy at a concentration of 20 mg, the correlation between BP and lipid profile in the group 3 was significantly improved compared to group 1 and group 2. SBP in group 3 was significantly related with measurement of DBP ($r = 0.896$, $p = 0.0001$), TG ($r = 0.513$, $p = 0.025$), % of HDL-C ($r = -0.507$, $p = 0.027$), and TG/HDL-C ($r = 0.589$, $p =$

TABLE 2 | Change of blood profile after 24 weeks policosanol consumption.

	Group 1 Placebo ($n = 24$, M14/F10)		Group 2 Policosanol 10 mg ($n = 29$, M21/F8)		Group 3 Policosanol 20 mg ($n = 31$, M22/F9)	
Blood profile	**Week 0**	**Week 24**	**Week 0**	**Week 24**	**Week 0**	**Week 24**
TC (mg/dL)	197 ± 10	184 ± 8	195 ± 7	180 ± 8*	185 ± 10	161 ± 7***
TG (mg/dL)	81 ± 7	91 ± 7	96 ± 13	97 ± 14	104 ± 17	97 ± 8
HDL-C (mg/dL)	39 ± 2	39 ± 3	36 ± 2	42 ± 3**	39 ± 2	44 ± 2*
% HDL-C in TC	21.4 ± 1.5	22.3 ± 1.3	18.8 ± 1.1	24.4 ± 2**	21.8 ± 1.9	28.0 ± 1.3***
TG/HDL-C	2.2 ± 0.2	2.1 ± 0.2	2.8 ± 0.5	2.5 ± 0.4	2.6 ± 0.6	2.2 ± 0.2
LDL-C (mg/dL)	132 ± 35	123 ± 27	131 ± 29	113 ± 25	126 ± 40	101 ± 30*
Glucose(mg/dL)	87 ± 13	85 ± 11	96 ± 31	90 ± 11*	92 ± 16	86 ± 12*
Renin (ng/mL/hr)	4.5 ± 1.4	3.9 ± 0.5	4.8 ± 1.1	3.1 ± 0.6*	6.5 ± 1.5	2.4 ± 0.4**
Aldosterone (ng/dL)	19.5 ± 1.2	19.0 ± 1.6	29.5 ± 2.3	19.6 ± 2.1*	24 ± 2.4	14 ± 1.5*
Angiotensin converting enzyme (unit)	33 ± 2	34 ± 2	31 ± 3	33 ± 4	34 ± 3	30 ± 3
Homocysteine (μmol/L)	14 ± 3	13 ± 3	14 ± 3	9 ± 1	13 ± 4	10 ± 2

*HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; LDL-C was calculated using Friedwald formula: LDL-C = TC-(HDL-C+TG/5) *p < 0.05; **p < 0.01; ***p < 0.001 versus week 0 in each group.*

TABLE 3 | Pearson's correlation analysis at the baseline and after 24 weeks in the placebo group (Group 1).

	At 0 week								At 24 weeks						
	SBP	**DBP**	**TC**	**TG**	**HDL-C**	**%HDL-C**	**TG/HDL-C**		**SBP**	**DBP**	**TC**	**TG**	**HDL-C**	**%HDL-C**	**TG/HDL-C**
SBP	1	0.798**	0.127	−0.630*	−0.096	−0.189	−0.427	SBP	1	0.574*	0.429	0.156	0.180	−0.146	−0.063
DBP		1	0.317	−0.415	−0.338	−0.514	−0.206	DBP		1	0.219	0.008	0.033	−0.155	−0.075
TC			1	−0.207	0.061	−0.639*	−0.241	TC			1	0.488	0.124	−0.503	0.322
TG				1	−0.518	−0.212	0.942**	TG				1	0.057	−0.292	0.754**
HDL-C					1	0.698**	−0.761**	HDL-C					1	0.786**	−0.584*
%HDL-C						1	−0.368	%HDL-C						1	−0.733**
TG/HDL-C							1	TG/HDL-C							1

*SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); TC, total cholesterol; TG, triglyceride (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); % HDL, percentage of high density lipoprotein cholesterol; TG/HDL-C, triglyceride ratio high density lipoprotein cholesterol. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).*

0.008) (**Table 5**). Moreover, DBP was positively related to TC ($r = 0.492$, $p = 0.032$), TG ($r = 0.497$, $p = 0.030$), and TG/HDL-C ($r = 0.549$, $p = 0.015$). A negative correlation was observed between DBP and % of HDL-C ($r = -0.0549$, $p = 0.015$).

Correlation of Central Aortic Blood Pressure With Lipid Parameters After 24 Weeks of Policosanol Therapy

In group 1, after 24 weeks of placebo therapy, central SBP was significantly correlated with central DBP. However, none of the lipid parameters (TG, TC, HDL-C, and % of HDL-C) showed a significant correlation with measurement of central SBP as well as central DBP (Table S1). After 24 weeks of therapy with 10 mg of policosanol (group 2), the correlation between central aortic pressure and the lipid profile improved slightly. Central SBP was significantly correlated with central DBP, TC, and % of HDL-C (Table S2). Moreover, central DBP was significantly correlated with TC, HDL-C, and % of HDL-C. HDL-C levels and % of HDL-C were negatively correlated with central DBP. In group 3, significance level in the correlation study was improved in comparison with group 1 and 2 and central SBP was significantly related to DBP. Additionally, central DBP was correlated with TG levels and TG/HDL-C (Table S3). Central DBP was also found to be significantly correlated with the concentration of TG and TG/HDL-C after 24 weeks of policosanol therapy.

LDL Oxidation

At week 0, all groups showed similar malondialdehyde contents in LDL, as shown in **Figure 2A**. However, at week 24, groups 2 and 3 showed 57 and 53% reductions in conjugated diene level, whereas group 1 showed 20% reduction. At week 0, LDL from all groups moved to the bottom of the gel with similar electromobilities (**Figure 2B**). Oxidized LDL moved faster to the cathode position due to an increased negative charge and apo-B fragmentation. At 24 weeks electromobilities of LDL from groups 2 and 3, were much slower compared with week 0, suggesting less production of negatively charged molecules and less fragmentation of apo-B in LDL.

Glycation Extent of Lipoproteins

After 24 weeks of policosanol consumption, the policosanol groups showed significantly lowered glycation extent in the HDL_2 and HDL_3 fractions, as shown in **Figure 3**. For the HDL_2

TABLE 4 | Pearson's correlation analysis at the baseline and after 24 weeks of consuming policosanol (10 mg) (Group 2).

	At 0 weeks								At 24 weeks						
	SBP	**DBP**	**TC**	**TG**	**HDL-C**	**%HDL-C**	**TG/HDL-C**		**SBP**	**DBP**	**TC**	**TG**	**HDL-C**	**%HDL-C**	**TG/HDL-C**
SBP	1	0.847**	0.132	0.511	−0.192	−0.202	0.478	SBP	1	0.555*	0.481	0.577*	−0.615*	−0.737**	0.700**
DBP		1	0.398	0.568*	−0.370	−0.487	0.553*	DBP		1	0.412	0.285	−0.434	−0.493	0.372
TC			1	0.406	−0.031	−0.618*	0.338	TC			1	0.266	−0.193	−0.703**	0.288
TG				1	−0.511	−0.619*	0.985**	TG				1	−0.392	−0.478	0.929**
HDL-C					1	0.794**	−0.627*	HDL-C					1	0.822**	−0.638*
%HDL-C						1	−0.672**	%HDL-C						1	−0.666*
TG/HDL-C							1	TG/HDL-C							1

*SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); TC, total cholesterol; TG, triglyceride (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); % HDL, percentage of high density lipoprotein cholesterol; TG/HDL-C, triglyceride ratio high density lipoprotein cholesterol. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).*

TABLE 5 | Pearson's correlation analysis at the baseline and after 24 weeks of consuming policosanol (20 mg) (Group 3).

	At 0 weeks								At 24 weeks						
	SBP	**DBP**	**TC**	**TG**	**HDL-C**	**%HDL-C**	**TG/HDL-C**		**SBP**	**DBP**	**TC**	**TG**	**HDL-C**	**%HDL-C**	**TG/HDL-C**
SBP	1	0.825**	0.257	0.485*	−0.460*	−0.433	0.487*	SBP	1	0.896**	0.402	0.513*	−0.235	−0.507*	0.589**
DBP		1	0.340	0.631*	−0.305	−0.387	0.593**	DBP		1	0.492*	0.497*	−0.119	−0.549*	0.549*
TC			1	0.585**	−0.389	−0.800**	0.555*	TC			1	0.181	−0.103	−0.881**	0.217
TG				1	−0.584**	−0.671**	0.988**	TG				1	0.117	−0.087	0.975**
HDL-C					1	0.832**	−0.670**	HDL-C					1	−0.512*	−0.097
%HDL-C						1	−0.703**	%HDL-C						1	−0.201
TG/HDL-C							1	TG/HDL-C							1

*SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); TC, total cholesterol; TG, triglyceride (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); % HDL, percentage of high density lipoprotein cholesterol; TG/HDL-C, triglyceride ratio high density lipoprotein cholesterol. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).*

fraction, groups 2 and 3 showed 44 and 40%, less production of AGEs, respectively, whereas the placebo group showed a similar level of glycation with an 8% increase (**Figure 3B**). For HDL_3, groups 2 and 3 showed 48 and 42% less production of AGEs respectively (**Figure 3B**). These results suggest that glycation extent of HDL was more reduced by policosanol consumption at both 10 and 20 mg.

SDS-PAGE and Western blot analysis for HDL_3 revealed that the apoA-I band position was slightly lower in after 24 weeks especially in groups 2 and 3, suggesting that electromobility was altered by policosanol consumption due to less glycation (**Figure 3C**). Immunodetection with apoA-I antibody revealed that apoA-I band intensities in groups 2 and 3 increased by 1.4-fold at week 24, compared with week 0 (**Figure 3D**). More interestingly, at week 0, groups 2 and 3 showed stronger band intensities for multimerized apoA-I such as dimer and trimer. However, multimeric band intensities were reduced at week 24, suggesting less glycation occurred in the policosanol group.

DISCUSSION

This study was a randomized clinical trial evaluating the effects of Cuban policosanol on BP and serum lipids in healthy participants who had pre-hypertension. The current results show that 24 weeks of policosanol consumption resulted in significant lowering of brachial (peripheral) BP and central aortic BP in a dose-dependent manner as well as lowering of serum renin and aldosterone levels in pre-hypertensive participants. For the blood lipid parameters, 24 weeks of policosanol therapy resulted in significant reduction of TC and LDL-C as well as concomitant increases in HDL-C and the HDL-C/TC ratio in a dose-dependent manner.

Castaño et al. (2002) reported the effect of policosanol on older patients with hypertension and type II hypercholesterolemia in a prospective, randomized, double-blind, placebo-controlled study. The participants consumed 5–10 mg/day of policosanol for one year, based on the total cholesterol values (6.1 mmol/L). Policosanol treatment group in a long-term significantly lowered the LDL-C (20.5%), TC (15.4%), TG (11.9%), LDL-C/HDL-C ratio (22.2%), and increased HDL-C up to 12.7%. Moreover, policosanol consumption significantly decreased systolic blood pressure when compared with the values of baseline and placebo (Castaño et al., 2002). Additionally, the efficacy and tolerability of policosanol in patients with high global coronary risk were revealed a significant decrease in serum LDL-C, TC, TG, TC/HDL-C, and LDL-C/HDL-C along with elevating the HDL-C. The treatment was well-tolerated; no drug-related clinical or biochemical adverse effects (AEs) were observed (Castano et al., 1999). The exact mechanism of policosanol on the

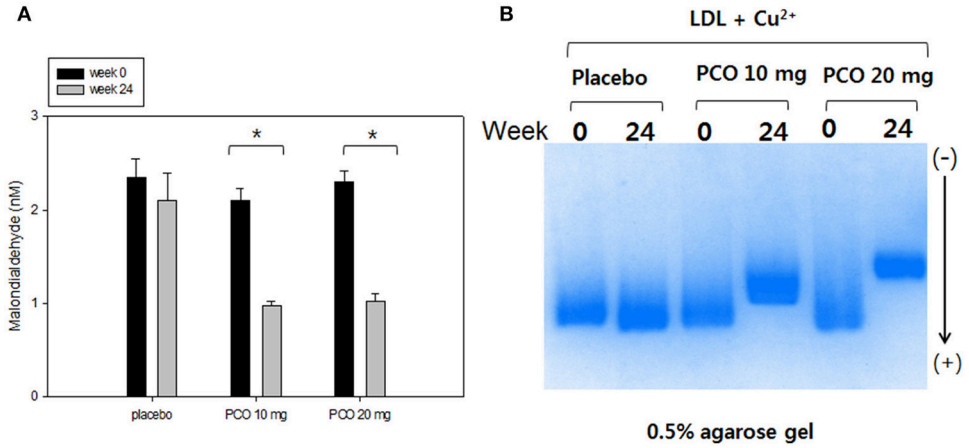

FIGURE 2 | Comparison of LDL oxidation extent before and after policosanol therapy. **(A)** Determination of oxidized species using thiobarbituric acid reactive substances method in LDL (1 mg of protein) in the presence of cupric ion at weeks 0 and 24. *$p < 0.05$ vs. week 0. **(B)** Comparison of electromobility of LDL between week 0 and 24 with cupric ion in 0.5% agarose gel.

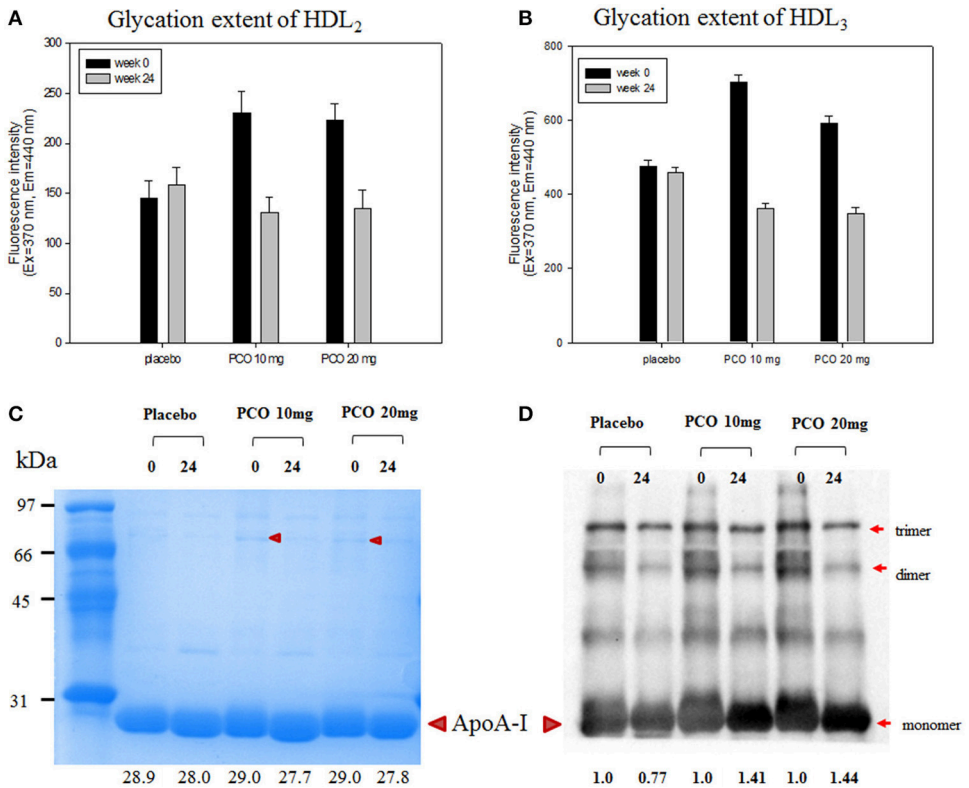

FIGURE 3 | Glycation extent and expression level of apoA-I in HDL_2 and HDL_3 at weeks 0 and 24. **(A)** Fluorometric determination (Ex = 370 nm, Em = 440 nm) of glycation extent at weeks 0 and 24 of HDL_2. **(B)** Fluorometric determination (Ex = 370 nm, Em = 440 nm) of glycation extent at weeks 0 and 24 of HDL_3. **(C)** Electrophoretic patterns of HDL_3 from each group at weeks 0 and 24 (5 μg/lane, 15% SDS-PAGE). Lower numbers indicate calculated molecular weight of apoA-I using Chemi-Doc (Bio-Rad). **(D)** Immunodetection of apoA-I in HDL_3 using anti-human apoA-I antibody (ab7613, Abcam). Lower numbers indicate calculated band intensity of apoA-I using Chemi-Doc (Bio-Rad).

lowering of lipid levels and blood pressure has not been clearly identified. However, some studies have evaluated that the action of policosanol involves various pathways such as activation of AMP-kinase, down—regulation of HMG-CoA reductase, and cholesteryl ester transfer protein inhibition (Mccarty, 2002; Singh et al., 2006; Kim et al., 2017).

Our current results indicate that the brachial BP lowering effect of policosanol is strongly correlated with TC, HDL-C, and the HDL-C/TC ratio in pre-hypertensive patients. More interestingly, the HDL-C/TC ratio (% HDL-C) showed a more negative correlation [correlation coefficient (r) = -0.737 $p < 0.001$] with reduction of SBP than HDL-C ($r = -0.615$, $p < 0.05$) in group 2. The TG/HDL-C ratio ($r = 0.700$, $p < 0.001$) was showed a strong positive correlation with SBP after policosanol therapy for 24 weeks than TG ($r = 0.577$, $p < 0.05$). The same tendency was observed in group 3, as % HDL (HDL-C/TC ratio) was more negatively correlated with SBP ($r = -0.507$, $p < 0.05$) along with DBP ($r = -0.549$, $p < 0.05$) after 24 weeks of therapy.

In placebo and participants consumed 10 mg of policosanol group, the lipid parameters and biomarkers did not vary when comparing values at baseline and after 24 weeks of therapy except for a significant lowering of total cholesterol in group 2 after 24 weeks of 10 mg of policosanol consumption. Participants consumed 20 mg of policosanol clearly showed improvement of the lipid profile (TC, HDL-C, % HDL-C in TC and LDL-C) after consumption of 20 mg of policosanol for 24 weeks. Within the same group, renin and aldosterone levels were reduced at 24 weeks compared to values at baseline. Previous studies reported a positive association of circulatory renin and aldosterone levels with hypertension (Tomaschitz et al., 2010a,b). Moreover, basic and clinical studies strongly suggested an association of the renin angiotensin-aldosterone system (RAAS) with pathogenesis of hypertension (Perticone et al., 2001; Atlas, 2007). Therefore, our study investigated the role of renin and aldosterone hormone in determining the plasma levels of the participants before and after policosanol therapy. Our study reported that consumption of 20 mg of policosanol for 24 weeks resulted in significant lowering of renin and aldosterone levels. There was a slight reduction in the levels of ACE in group 3 after 24 weeks, although the change was not significant. The concentration of homocysteine was not altered in any group after 24 weeks of therapy. This result is corroborated by a previous finding in which participants with metabolic syndrome showed no direct relationship between homocysteine and RAAS (Zacharieva et al., 2008). However, homocysteine levels are independently associated with risk of causing hypertension, as demonstrated from previous studies (Wang et al., 2014; Yang et al., 2017).

In addition to the consideration of brachial and central aortic BP, we measured mean arterial pressure (MAP) in this study which defines the average arterial pressure during a single cardiac cycle and was estimated using the formula (SBP + 2 × DBP)/3. Our study evaluated the MAP, which is more advantageous in diagnosing hypertension and related morbidity. Group 1 did not show any significant difference in MAP after 24 weeks of study. However, after consumption of policosanol at 10 mg and 20 mg per day for 24 weeks, groups 2 and group 3 showed MAP levels of 97 ± 11 mmHg ($p < 0.05$) and 93 ± 9 mmHg ($p < 0.001$) respectively. This result shows that policosanol not only affected central BP, but also helped to alleviate MAP. A higher MAP is correlated with higher incidence of hypertension (Sesso et al., 2000; Yadav et al., 2017) and metabolic syndrome (Hsu et al., 2015). Regarding the importance of aortic BP measurement, while brachial BP is determined by cardiac output and peripheral vascular resistance, central aortic pressure additionally determined by the stiffness of the conduit vessels and the timing/magnitude of pressure wave reflections (Mitchell et al., 2003).

Consumption of policosanol for 24 weeks resulted in significant reduction of central aortic SBP and DBP, indicating that policosanol could lower burden on the left ventricle and risk of CVD. Central aortic pressure is subjected to higher alteration by allopathic drugs than peripheral BP. For management of hypertension, antihypertensive drugs show differential effects on central pressure compared to their effects on brachial pressure (Morgan et al., 2004; Williams et al., 2006; Mackenzie et al., 2009). Dhakam et al. (2006) reported that inhibitors of the RAAS have a significantly greater reduction effect on central pressure compared to brachial pressure (Dhakam et al., 2006). In the REASON trial, normalization of brachial SBP was achieved along with significant greater reduction of central SBP using a low-dose combination of perindopril/indapamide therapy but not atenolol (London et al., 2004). Hypertension treatment based on central BP is more useful than brachial pressures as discussed in recent reports (Sharman et al., 2013; McEniery et al., 2014).

We previously reported that policosanol could inhibit glycation and oxidation *in vitro* (Lee et al., 2016) and *in vivo* (Kim et al., 2017). It is well-known that oxidized LDL is an independent risk factor for CVD and metabolic syndrome (Holvoet et al., 2008). OxLDL is a potent inflammatory trigger of atherosclerosis and vascular complications. Policosanol consumption at both 10 and 20 mg resulted in less production of oxLDL (**Figure 2**). Few reports on policosanol administration in animals such as in rat and rabbits exhibited a beneficial effects on plaque composition, stability and preventive effects on atherosclerotic lesions including form cell formation (Noa et al., 1995, 1996; Arruzazabala et al., 2000). However, the beneficial effects of policosanol on atherosclerosis development in human remain to be elucidated and future studies are needed to explore whether policosanol reduces the progression of atherosclerosis.

The pathological mechanism of glycation is initiated by non-enzymatic attachment of carbohydrates to blood proteins, especially hemoglobin, and lipoproteins (Nawale et al., 2006). It is well-known that glycation promotes development of aging and metabolic syndrome, including premature atherosclerosis via damage of key proteins. Glycation reduces stability of apoA-I, causing impairment of functionality in type 2 diabetes patients (Kashyap et al., 2017). Policosanol consumption remarkably lowered glycation extent in both HDL_2 and HDL_3 (**Figures 3A,B**) along with less multimerization of apoA-I. Since multimerization of apoA-I is directly associated with aggregation and the amyloidogenic process, dysfunctional HDL is more rapidly produced. Glycation of HDL also triggered more oxidative stress and blood vessel stiffness, resulting in incidence of hypertension. However, policosanol consumption also inhibited multimerization of apoA-I and increased content of content of monomeric apoA-I accompanied by a lower molecular weight band position (**Figure 3D**). Since glycated apoA-I showed higher molecular weight with smear band intensity as in our previous report (Park et al., 2010), clearer band intensity along with a lower molecular weight band position for apoA-I at week 24 indicates that glycation of HDL was prevented by policosanol consumption.

In conclusion, 24 weeks of policosanol consumption significantly reduced BP accompanied by enhancement of the lipid profile and lipoprotein properties, including reduction of TC, TG/HDL-C, and LDL-C as well as elevation of HDL-C and % HDL-C, resulting in improved anti-oxidant and anti-glycation activities. These results suggest that treatment of hypertension is possible via improvement of the lipid profile and lipoprotein functionality by policosanol therapy.

AUTHOR CONTRIBUTIONS

S-JK and DY: Performed experiments; H-JP and J-RK: Analyzed data; K-HC: Wrote the manuscript and supervised the whole project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00412/full#supplementary-material

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REVIEW
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Antioxidant Melatonin: Potential Functions in Improving Cerebral Autoregulation After Subarachnoid Hemorrhage

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Subarachnoid hemorrhage (SAH) is a subtype of stroke with high mortality and morbidity. Impaired cerebral autoregulation following SAH has been reported owing to effects on sympathetic control, endothelial function, myogenic response, and cerebral metabolism. Impaired cerebral autoregulation is associated with early brain injury, cerebral vasospasm/delayed cerebral ischemia, and SAH prognosis. However, few drugs have been reported to improve cerebral autoregulation after SAH. Melatonin is a powerful antioxidant that is effective (easily crosses the blood brain barrier) and safe (tolerated in large doses without toxicity). Theoretically, melatonin may impact the control mechanisms of cerebral autoregulation via antioxidative effects, protection of endothelial cell integrity, suppression of sympathetic nerve activity, increase in nitric oxide bioavailability, mediation of the myogenic response, and amelioration of hypoxemia. Furthermore, melatonin may have a comprehensive effect on cerebral autoregulation. This review discusses the potential effects of melatonin on cerebral autoregulation following SAH, in terms of the association between pharmacological activities and the mechanisms of cerebral autoregulation.

Keywords: melatonin, cerebral autoregulation, subarachnoid hemorrhage, antioxidant, sympathetic nerve, endothelial function

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INTRODUCTION

Cerebral autoregulation is defined as the mechanism by which constant cerebral blood flow is maintained, despite changes in arterial blood pressure (Guo et al., 2016). In the cerebral arterial system, cerebral autoregulation has been reported to be involved in all types of stroke and is related to secondary brain injury and prognosis (Vavilala et al., 2003; Chen et al., 2014b; Guo et al., 2014; Ma et al., 2016). In the reviews of Paulson and Strandgaard in 1984 and 1990, they concluded that the regulating mechanisms of cerebral autoregulation are including sympathetic control, cerebral metabolism, endothelial function and myogenic response (Strandgaard and Paulson, 1984; Paulson et al., 1990). Later, Bailey proposed that oxidative stress is also associated with impaired cerebral autoregulation and blood-brain barrier leakage (Bailey et al., 2011).

Subarachnoid hemorrhage (SAH) is a subtype of stroke with high mortality and significant morbidity. Delayed cerebral vasospasm and delayed cerebral ischemia are among the primary causes of poor prognosis following SAH. Cerebral autoregulation has been reported to be

impaired after SAH, and this phenomenon is associated with cerebral vasospasm/delayed cerebral ischemia (Budohoski et al., 2012, 2013; Otite et al., 2014; Calviere et al., 2015; Guo et al., 2016; Santos et al., 2016; Gaasch et al., 2018). Thus, cerebral autoregulation may be a potential therapeutic target for improving prognosis after SAH.

Melatonin is a hormone secreted by the pineal gland during the dark phase of the light-dark cycle, which is modulated by light-dark cycle (Bruls et al., 2000). Besides the pineal gland, melatonin was also produced in bone marrow (Tan D.X. et al., 1999). In addition, Tan D. et al. (1999) found high levels of melatonin in the bile of mammals of unknown origin. Previous studies reported that melatonin is a powerful antioxidant, which is known to be effective (it easily crosses the blood brain barrier) and safe (non-toxic in high doses) (Reiter et al., 2000). It has been studied in several cerebrovascular diseases, including ischemic stroke (Beker et al., 2015; Feng et al., 2017), intracerebral hemorrhage (Li et al., 2009; Lekic et al., 2010), and SAH (**Table 1**) (Fang et al., 2009; Wang et al., 2012, 2013; Chen et al., 2014a,c, 2015; Dong et al., 2016; Zhao et al., 2016), with respect to the mechanisms of antioxidation (Garcia et al., 2014; Zhang and Zhang, 2014; Manchester et al., 2015) and anti-inflammation (Agil et al., 2013; Mauriz et al., 2013; Chen et al., 2014a; Yang et al., 2014; Liu et al., 2015; Dong et al., 2016). These pharmacological activities of melatonin also potentially improve cerebral autoregulation after SAH. The present review discusses the potential effect of melatonin on cerebral autoregulation after SAH with respect to the association between pharmacological activities and mechanisms regulating cerebral autoregulation.

CEREBRAL AUTOREGULATION DYSFUNCTION AFTER SAH

Clinical Findings

Recently, an increasing number of studies have focused on the relationship between cerebral autoregulation and SAH and have reported that the impairment of cerebral autoregulation is related to poor prognosis after SAH. Otite et al. (2014) reported that patients who developed delayed cerebral vasospasm and delayed cerebral ischemia after SAH had worse cerebral autoregulation than did those who did not develop either of the conditions. Budohoski et al. (2015) conducted a study to determine the underlying consequences of unilateral and bilateral cerebral autoregulation damage on outcomes in SAH patients. They found that unilateral and bilateral cerebral autoregulation damage was related to delayed cerebral ischemia and unfavorable outcomes, respectively (Budohoski et al., 2015). Santos et al. (2016) analyzed the pathophysiological basis of the impairment of cerebral autoregulation in SAH and its relationship to prognosis. They found that cerebral autoregulation was significantly impaired in SAH patients who developed delayed cerebral ischemia compared with those who did not develop secondary brain injury or cerebral vasospasm alone (Santos et al., 2016). Similar results were reported by several studies (**Table 2**) (Lang et al., 2001; Soehle et al., 2004; Tseng et al., 2006; Budohoski et al., 2012, 2013, 2016; Calviere et al., 2015; Gaasch et al., 2018).

Mechanisms

Impaired cerebral autoregulation after SAH is possibly caused by oxidative stress, endothelial dysfunction, sympathetic activation, myogenic response disorder, and abnormal cerebral metabolism. A detailed study of these mechanisms might lead to future therapeutic possibilities.

Oxidative Stress After SAH

After SAH, oxidative stress is implicated in the etiology of at all stages of SAH (early brain injury, cerebral vasospasm, and delayed cerebral ischemia) (Ersahin et al., 2010; Zhang et al., 2015; Li et al., 2016; Ye et al., 2018). The high concentration of reactive oxygen species (ROS)/reactive nitrogen species (RNS) is considered to be associated with impaired cerebral autoregulation (Choi et al., 2001; Shin et al., 2002) (**Figure 1**). One important mechanism has been reported to result in impaired cerebral autoregulation is because of the direct and indirect actions of ROS/RNS on K+ channels. The K+ channels, including ATP-sensitive K+ channels and large conductance Ca^{2+}-activated K^{+} channels, can regulate the activation and contraction of cerebral arterial muscle cells, and subsequently change the smooth muscle tone (Lee et al., 1993; Nelson and Quayle, 1995; Shin and Hong, 2004; Zagorac et al., 2005). Moreover, the high concentration of free radicals may cause impaired cerebral autoregulation in several other pathways, including damaged endothelial cells function (followed by integrity destroyed and nitric oxide availability reduced), and inducted inflammatory response (followed by endothelial cells dysfunction and hypoxemia condition). These factors are discussed in the following sections (**Figure 1**).

Endothelial Dysfunction After SAH

The vascular endothelial mechanism is an essential part of cerebral autoregulation because endothelial cells modulate many aspects of vascular functioning, particularly in controlling the vascular tone (Pries et al., 2000; Rodella et al., 2013). The structural and functional integrity of endothelial cells is essential for maintaining stable cerebral autoregulation (Preckel et al., 1996; White et al., 2000; Ainslie et al., 2007; Guo et al., 2016). After SAH, both structural and functional integrity were damaged because of factors, such as the high concentration of ROS/RNS and inflammatory responses (**Figure 1**) (Kajita et al., 1998; Scharbrodt et al., 2009; Szatmari et al., 2010; Sabri et al., 2011; Qin et al., 2012; Liu et al., 2016; de Azevedo et al., 2017; Shekhar et al., 2017; Armstead et al., 2018). In inflammatory responses, various inflammatory pathways, such as the NF-κB pathway (Pawlowska et al., 2018), NLRP3 pathway (Li et al., 2016; Shao et al., 2016), and TLR4 pathway (Zhang et al., 2016), are activated and have negative effects on the arterial endothelium after SAH. In the downstream of these pathways are inflammatory factors, interleukin-1β, and tumor necrosis factor-α. These inflammatory factors act on vascular endothelium, resulting in changes in the concentration and bioavailability of endothelium-derived nitric oxide.

Nitric oxide, the most important vasodilation factor, can regulate the vascular tone of small arteries; the mechanism is that nitric oxide diffuses into the adjacent smooth muscle cells

TABLE 1 | Functions of melatonin in improving brain injury after subarachnoid hemorrhage.

Journal	First author	Year	Action targets
J Pineal Res	Dong Y	2016	Regulating NLRP3 inflammasome and apoptosis signaling.
Mol Neurobiol	Zhao L	2016	Regulating melatonin receptor/Sirt1/NF-κB signaling pathway
J Pineal Res	Chen J	2014	Regulation of pro-inflammatory cytokines
J Pineal Res	Chen J	2014	Regulating mitochondrial pathway
J Pineal Res	Wang Z	2013	Regulating TLR4-mediated inflammatory pathway
J Pineal Re	Wang Z	2012	Activating the Nrf2-ARE pathway
Mediators Inflamm	Fang Q	2009	Regulating nuclear factor-kappa pathway and proinflammatory cytokines expression

TABLE 2 | Literature on cerebral autoregulation (CA) and subarachnoid hemorrhage in humans.

Journal	First author	Year	Main outcomes
Crit Care Med	Gaasch M	2018	CA was associated with delayed cerebral ischemia (DCI) and poor functional outcome
Neurology	Santos GA	2016	CA can predict neurologic complications
Acta Neurochir Suppl	Budohoski KP	2016	Impaired CA in the first 5 days after SAH is predictive of DCI
Neurocrit Care	Calviere L	2015	Early deterioration of CA was strongly predictive of DCI
Neurocrit Care	Budohoski KP	2015	Unilateral CA failure was seen in patients who developed DCI, and bilateral CA failure was seen more frequently in patients with unfavorable outcome
Stroke	Otite F	2014	Impaired CA is associated with vasospasm and DCI
J Cereb Blood Flow Metab	Budohoski KP	2013	CA can aid in predicting DCI
Stroke	Budohoski KP	2012	Disturbed CA in the first 5 days after SAH significantly increases the risk of DCI
Neurosurg Focus	Tseng MY	2006	CA may help identify patients at high risk of delayed ischemic neurological deficits.
Anesth Analg	Soehle M	2004	CA was impaired during cerebral vasospasm
Crit Care Med	Lang EW	2001	CA impairment precedes vasospasm, and ongoing vasospasm worsens CA

and relaxes them by increasing cyclic guanosine monophosphate (Kajita et al., 1998). Because of the physiological effects of nitric oxide, Guo et al. (2016) proposed that reduced nitric oxide availability may relate to the impaired cerebral autoregulation after SAH due to endothelium-dependent mechanism. The results reported by Tseng et al. (2005) support this hypothesis; these authors found that pravastatin, a member of the drug class of statins, can improve vascular endothelium-dependent relaxation to acetylcholine and increase endothelial nitric oxide synthase activity, as well as improve cerebral autoregulation after SAH (Tseng et al., 2005; Yamamoto et al., 2007). Thus, these studies indicated that the improvement in endothelial function is a therapeutic target to improve cerebral autoregulation.

Sympathetic Activation After SAH

The cerebrovascular bed is innervated by sympathetic nerve fibers (Edvinsson et al., 1975; Hamner et al., 2010). The sympathetic nervous system regulates cerebral blood flow by managing cerebral vascular resistance (Aubineau et al., 1980; Busija, 1985; Guo et al., 2016). Theoretically, after the stimulation of sympathetic nervous system, alpha-1 adrenergic receptors are activated by norepinephrine released by post-ganglionic sympathetic neurons, resulting in vasoconstriction (Aubineau et al., 1980; Busija, 1985; Guo et al., 2016). Several studies have reported that sympathetic control plays an important role in regulating cerebral autoregulation, and that sympathetic dysfunction can cause impaired cerebral autoregulation (Sadoshima et al., 1985; Hamner and Tan, 2014; Guo et al., 2016). Notably, the acute stage of SAH is accompanied by significant sympathetic activation (Moussouttas et al., 2012b, 2014). Sympathetic activation results in increased concentration of circulating catecholamines (epinephrine, noradrenaline, and serotonin), which are associated with cerebral vasospasm and delayed cerebral ischemia after SAH (Grad et al., 1991; Dilraj et al., 1992; Naredi et al., 2000; Banki et al., 2005; Moussouttas et al., 2012a). The vasoconstriction of blood vessels caused by sympathetic activation after SAH is a possible mechanism underlying cerebral autoregulation dysfunction (**Figure 1**).

Myogenic Response Disorder After SAH

Smooth muscle is a main component of cerebral arteries. The control of arterial myogenic tone was first described by Bayliss (1902). The myogenic response was regulated by a complex mechanism, and some of these mechanisms are out of balance after SAH (Lidington et al., 2018). (1) Previous study reported that potassium channels are important regulators of vascular tone. SAH can reduce potassium currents in cerebral artery smooth muscle cells and then enhanced constriction (Jahromi et al., 2008). (2) After SAH, endothelial dysfunction was observed, resulting in reduced vasodilating factors levels. Thus, it is reasonable to speculate that endothelial dysfunction augments myogenic response disorder (Lidington et al., 2018). (3) The effect of ROS on myogenic response disorder was our concern. ROS are believed to be involved in cellular signaling in blood vessels, and to directly and indirectly mediate vascular smooth muscle via regulating endothelium-dependent contractions pathway (Cosentino et al., 1994), and calcium-activated potassium channels (Wei et al., 1996; Faraci,

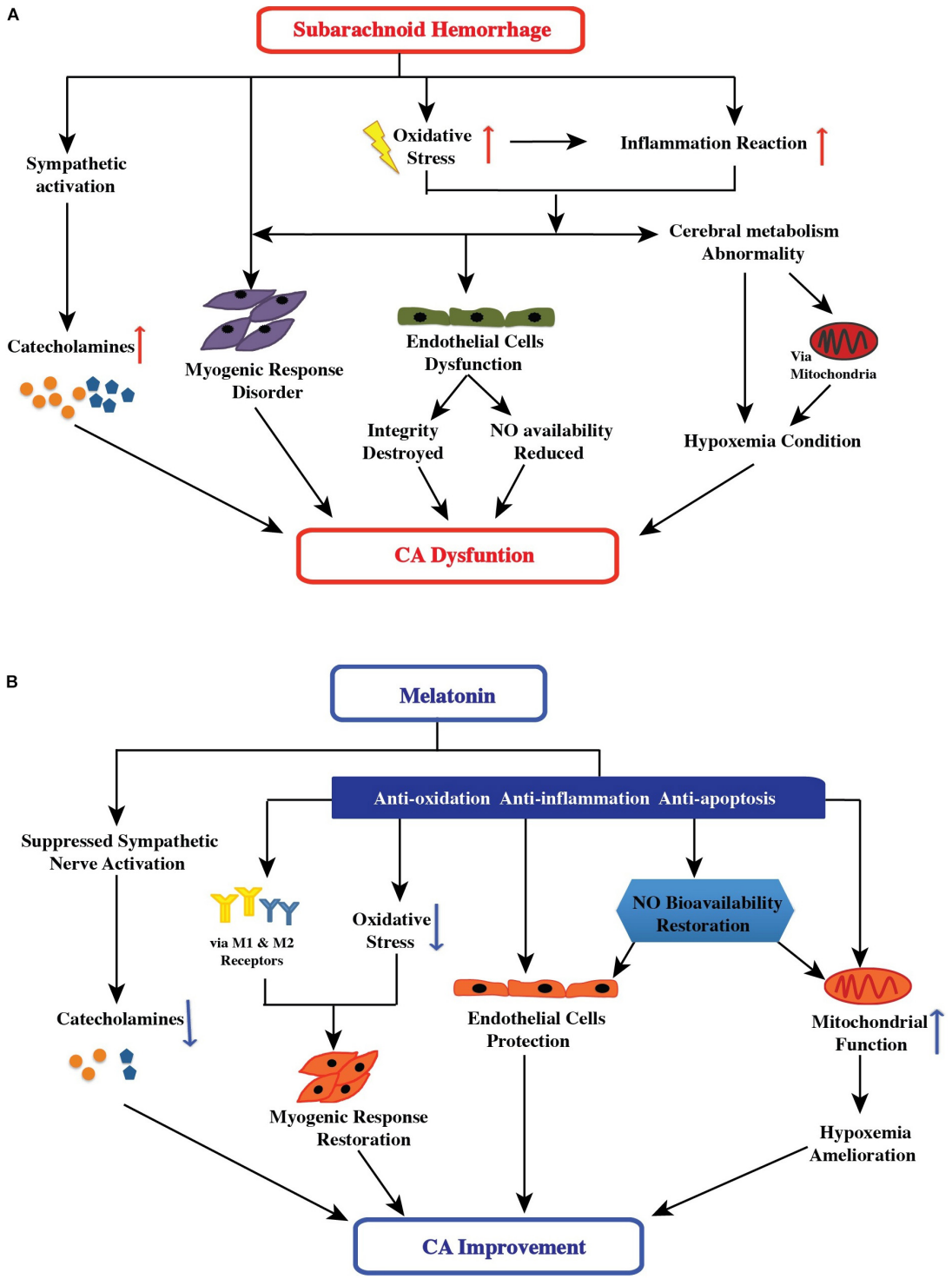

FIGURE 1 | (A) Effect of subarachnoid hemorrhage on the control mechanisms of cerebral autoregulation. After subarachnoid hemorrhage, oxidative stress is implicated in the etiology of all stages: the high free-radical concentration leading to impaired cerebral autoregulation has direct and indirect effects, including changing smooth muscle tone, damaging endothelial cell function (followed by destruction of endothelial cell integrity and reduction of nitric oxide availability), and inducing an inflammatory response (followed by endothelial cell dysfunction and hypoxemia). Additionally, sympathetic activation and abnormal cerebral metabolism after SAH aggravated the impairment of cerebral autoregulation. **(B)** Possible therapeutic targets of melatonin in improving cerebral autoregulation after subarachnoid hemorrhage. After subarachnoid hemorrhage, melatonin may potentially impact the control mechanisms of cerebral autoregulation via suppression of sympathetic nerve and antioxidant activity, increase in nitric oxide bioavailability, direct and indirect mediation of the myogenic response, and amelioration of hypoxemia. Furthermore, melatonin can have a comprehensive effect on cerebral autoregulation.

2006). Previous studies also reported that both relaxation and contraction of the vascular muscle were caused by ROS, which may dependent on concentrations (Faraci, 2006). For instance, Rosenblum found that the generation of superoxide using acetaldehyde and xanthine oxidase produces dilation of cerebral arterioles at low substrate concentrations, but vasoconstriction at higher substrate concentrations followed by dilation (Rosenblum, 1983). Studies *in vivo* found that hydrogen peroxide acts as a vasodilator on small cerebral arteries via activated potassium channels. However, high concentrations of hydrogen peroxide can produce vasoconstriction followed by vasodilation (Faraci, 2006). The general trend is that, ROS produces vasodilation at low concentrations and vasoconstriction at higher concentrations (Rosenblum, 1983; Cosentino et al., 1994; Wei et al., 1996; Faraci, 2006). Thus, high concentration of ROS (derived from blood) may augment the myogenic tone via directly and indirectly mediate vascular smooth muscle after SAH. Recently, a study from Deng et al. (2018) provided direct evidence. They studied the effects of extravascular hemolyzed blood on arteriolar myogenic constriction and found that extravascular hemolyzed blood augments the myogenic constriction of cerebral arterioles, possibly by increasing the vascular production of superoxide. In addition to ROS pathways, a study reported that tumor necrosis factor-α/sphingosine-1-phosphate signaling can augment the myogenic tone in experimental SAH mouse model (Yagi et al., 2015). These studies indicated that the effects of the myogenic response on cerebral autoregulation may be caused multiple pathways and need further analysis.

Metabolism Abnormal Impaired Cerebral Autoregulation After SAH

Under physiological conditions, when the cerebral blood volume was decreased, some vasoactive substances were released from the brain, caused the cerebral arteries to become dilated, and vice versa. This phenomenon was considered a metabolic mechanism of cerebral autoregulation to maintain stable cerebral perfusion. Actually, apart from vasoactive substances, oxygen and carbon dioxide levels and metabolites can regulate cerebral autoregulation. After SAH, the reduced brain tissue oxygen pressure and brain pH (Carvi y Nievas et al., 2005) can change cerebral microcirculation and metabolism, perhaps partly because of oxidative damage (Lopez et al., 2009). These changes may cause cerebral autoregulation dysfunction (**Figure 1**).

THE ROLE OF MELATONIN IN IMPROVING CEREBRAL AUTOREGULATION AFTER SAH

Melatonin Improves Cerebral Autoregulation by Antioxidation

Previous studies have found that melatonin and its metabolites (mainly N^1-acetyl-N^2-formyl-5-methoxykynuramine and *N*-acetyl-5-methoxykynuramine) are powerful free radical scavengers, and scavenge various types of free radicals, such as hydroxyl radicals and hydrogen peroxide. Through a cascade reaction involving melatonin and its metabolites, a melatonin molecule can scavenge up to 10 ROS/RNS (Tan et al., 2002; Zavodnik et al., 2006; Hardeland et al., 2007; Tan et al., 2007). Besides, Boussard et al. (2006) reported that the third melatonin binding site (MT3), characterized as the enzyme quinone reductase 2, may contribute to melatonin antioxidant properties by inhibiting the electron transfer reactions of quinones (Boussard et al., 2006; Pandi-Perumal et al., 2008; Emet et al., 2016).

In addition, melatonin plays an important role in activating antioxidant defenses. Venkataraman et al. (2010) found that exogenous melatonin supplementation can rescue the decreased mRNA expression of Cu/Zn superoxide dismutase and glutathione peroxidase-4 in a polychlorinated biphenyl-induced neuronal damage rat model. Akcay et al. (2005) reported that treatment with exogenous melatonin can maintain malondialdehyde levels and catalase and superoxide dismutase activities at normal levels in the brain cortex of a kainic acid-induced injury rat model. Moreover, melatonin can inhibit pro-oxidant enzymes, such as inducible nitric oxide synthase (Lopez et al., 2006; Kang et al., 2013).

Thus, melatonin is a useful antioxidant, and acts via multiple antioxidant pathways; its antioxidative actions directly and indirectly improve cerebral autoregulation by protecting endothelial function and increasing nitric oxide bioavailability, mediating myogenic responses, and ameliorating conditions of hypoxemia. This is discussed in the following sections.

Melatonin Improves Cerebral Autoregulation by Protecting Endothelial Function and Increasing Nitric Oxide Bioavailability

As mentioned above, melatonin serves as an antioxidant via several pathways. It also has anti-inflammatory effects. Several studies have reported the possible pathways through which melatonin attenuates inflammation in the brain (Carrascal et al., 2018; Wang et al., 2018). Jumnongprakhon et al. (2016) found that melatonin can prevent methamphetamine-induced inflammatory responses by inhibiting the nuclear factor-κB pathway and promoting the nuclear factor erythroid 2-related factor-2 pathway before blood-brain barrier impairment. Dong et al. (2016) suggested that melatonin regulates the NLRP3 inflammasome pathway, and thus attenuates early brain injury after SAH. Wang et al. (2013) proposed that melatonin can alleviate secondary brain damage through the TLR4-mediated inflammatory pathway after SAH. Fu et al. (2017) also reported that melatonin supplementation may be a valuable therapeutic strategy in cases of inflammatory neurological dysfunction, and that melatonin may subserve this function through the inhibition of TLR4 signaling. Zhao et al. (2015) reported that melatonin attenuates sepsis-induced brain injury by activating silent information regulator 1 signaling (Zhao et al., 2015). In addition, oxidative stress plays a vital role in mediating the initial phase of the inflammatory reaction by regulating leukocyte recruitment and maturation and activating intracellular inflammatory pathways, resulting in increased

levels of various inflammatory mediators (Cristofanon et al., 2009; Radogna et al., 2010). Melatonin can regulate signaling through these pathways and thus inhibit inflammatory processes (**Figure 1**).

Previous studies have reported that melatonin functions to increase nitric oxide bioavailability. Aladag et al. (2009) conducted a study in an SAH rat model and showed that the administration of melatonin ameliorates cerebral vasospasm via an increase in serum nitric oxide concentration and a decrease in the levels of arginase and oxidative stress in the brain. Similarly, using intermittent hypoxia rat models, Tjong et al. (2008) showed that melatonin ameliorates constitutive nitric oxide production and large conductance calcium-activated potassium channel activity through an antioxidant pathway. Wakatsuki et al. (2001) studied the antioxygenation effect of melatonin on the oxidized low-density lipoprotein-induced impairment of nitric oxide production, and found that pre-treatment with melatonin reversed the oxidized low-density lipoprotein-induced reduction in nitric oxide production. In their review, Simko and Paulis noted that melatonin may increase nitric oxide levels via the promotion of nitric oxide production and/or the prevention of coupling to the superoxide anion radical (Simko and Paulis, 2007). However, some studies have reported that melatonin reduces nitric oxide levels in middle cerebral artery occlusion stroke rat models (Pei et al., 2003) and cerebral ischemia/reperfusion Mongolian gerbil models (Guerrero et al., 1997). Thus, the role of melatonin in nitric oxide production requires further investigation.

Previous studies show that melatonin can act as an endothelial protective agent via the disruption of oxidative stress and inflammatory response pathways and may also regulate nitric oxide concentration and bioavailability. Thus, it can protect the integrity and function of vascular endothelial cells.

Melatonin Improves Cerebral Autoregulation by Suppressing Sympathetic Nerve Activity

In previous studies, melatonin has been shown to regulate sympathetic nerve activity. Viswanathan et al. (1986) found that the administration of melatonin to Syrian hamsters suppressed the sympathetic nervous system. Cagnacci et al. (1998) found that the administration of melatonin decreased blood pressure and blunt noradrenergic activation in young women. Arangino et al. (1999) reported that oral administration of melatonin could reduce blood pressure, vascular reactivity, and norepinephrine levels in men. Girouard et al. (2004) found that exogenous melatonin improved the baroreflex response associated with improved antioxidation in spontaneously hypertensive rats, suggesting a correlation between antioxidation and the decreased sympathetic tone induced by melatonin. In another study of spontaneously hypertensive rats, K-Laflamme et al. (1998) found that after 20 min of melatonin administration, the plasma epinephrine concentration reduced by approximately 60%, and the norepinephrine concentration decreased by approximately 30%. This indicated that the action of melatonin involved the inhibition of basal sympathoadrenal tone (K-Laflamme et al., 1998). Interestingly, Olmez and Kurcer (2003) found that melatonin can attenuate alpha-adrenergic-induced contractions by increasing vasoactive intestinal peptide levels in isolated rat penile bulbs. In addition, several studies have found that melatonin can affect the neural control of reflex changes in muscles and sympathetic nerve activity in the skin (Ray, 2003; Muller et al., 2013). Hence, the role of melatonin in regulating sympathetic nerve activity is gradually becoming clearer. However, these evidences of melatonin in regulating sympathetic nerve activity is based on systemic effects, we also tried to find direct evidence of melatonin on cerebral regulation. After careful searching, only one study was found. Bang et al. (2012) reported that exogenous melatonin did not affect the cardiovascular reflex and dynamic cerebral autoregulation responses to acute hypotension in twelve healthy men. The reason for the negative results may due to the subjects were healthy adults and the sample size was too small. Thus, theoretically, melatonin may be a potentially useful drug for improving cerebral autoregulation via a reduction in sympathetic nerve activity after SAH (**Figure 1**), but the actual effect of melatonin remains to be studied.

Melatonin Improves Cerebral Autoregulation by Mediating Myogenic Response

As mentioned in the previous section, the ROS concentration may play an important role in regulating vasomotor function after SAH. Melatonin acts as a powerful ROS scavenger, functioning to mediate myogenic responses after SAH. In addition, exogenous melatonin reduces the concentration of tumor necrosis factor-α in the brain (Pazar et al., 2016; Taniguti et al., 2018), and may thus reduce tumor necrosis factor-α-mediated myogenic tone augmentation (Yagi et al., 2015). Weekley (1991) reported that melatonin induces the dose-dependent relaxation of precontracted vascular smooth muscle of rat aorta, and this response was not affected by vascular endothelium removal.

Furthermore, melatonin can have direct effects on smooth muscle through its receptors. Humans have two plasma membrane receptors of melatonin, MT1 and MT2, which are expressed in various tissues, including brain, retina, cardiovascular system, and liver tissues (Ekmekcioglu, 2006). MT1 and MT2 belonging to the G-protein-coupled receptor superfamily, which constitutes adenylate cyclase inhibition by binding to various G-proteins (Pandi-Perumal et al., 2008; Emet et al., 2016). In the central nervous system in humans, melatonin receptors are observed in suprachiasmatic nuclei (Weaver and Reppert, 1996), retina (Reppert et al., 1995; Thomas et al., 2002; Ekmekcioglu, 2006), hippocampus (Savaskan et al., 2002; Savaskan et al., 2005), and cerebellar cortex (Al-Ghoul et al., 1998). In studies on the caudal artery, the MT1 receptor mRNA was primarily found in the smooth muscle layer, whereas the MT2 receptor mRNA appeared more evenly distributed throughout the vessel wall. However,

both MT1 and MT2 in vascular smooth muscle cells can regulate the vascular tone. Doolen et al. (1998) indicated that MT1 receptor activation may mediate vasoconstriction. Subsequently, Lew and Flanders (1999) further indicated that melatonin elicited the contraction of the rat tail artery by activating an MT1 receptor that coupled to the activated L-type calcium channels. For the MT2 receptor, study conclusions are inconsistent. Masana et al. (2002) indicated that after using MT2 antagonists, the melatonin-mediated vasocontraction was enhanced, indicating MT2 receptors located in vascular smooth muscle mediate vasodilation. Similarly, Doolen et al. (1998) also found MT2 receptors may induce relaxation. However, a study reported that MT2 receptor activation in coronary vascular smooth muscle cells is associated with inhibiting nitric oxide-induced increases in cyclic GMP and coronary arterial relaxation (Tunstall et al., 2011). The comprehensive effects of melatonin receptors in regulating the myogenic response warrants further studies. There are also studies of the effect of melatonin on cerebral arteries. Régrigny et al. (1999) found that melatonin can induce the increase of cerebral arteriolar tone via stimulating MT1 and/or MT2 receptors followed by blockade of calcium-activated large conductance potassium channels in rats, they also reported that melatonin decreased the lower limit of cerebral blood flow autoregulation, which may potential reduce the risk of hypoperfusion-induced cerebral ischemia. Later, Lapi et al. (2011) studied the rat pial microvascular responses induced by melatonin during brain hypoperfusion and reperfusion injury. They found melatonin can regulate the pial arteriolar tone and then promote an efficient redistribution of microvascular blood flow via activating MT1 and MT2 receptors, they further reported that lower dosage of melatonin stimulate MT2 receptors, while higher dosage activated also MT1 receptors (Lapi et al., 2011). From the above two studies, we can speculate that melatonin may have neuroprotective effect via regulating myogenic response of cerebral arteries.

Melatonin Improves Cerebral Autoregulation by Ameliorating Hypoxemia and Regulating Metabolism

To ameliorate hypoxemia, mitochondrial function is crucial. Melatonin can protect mitochondrial functioning through its anti-apoptosis, antioxidative, and combined anti-apoptosis and antioxidative effects. Lopez et al. (2009) have indicated that melatonin protects mitochondria from damage due to oxidative stress by reducing oxygen consumption, membrane potential, and superoxide anion production. Carretero's study presents the same conclusions (Carretero et al., 2009). Yamamoto and Mohanan concluded that melatonin protects against attenuated brain mitochondrial DNA damage induced by hydroxyl radicals (Yamamoto and Mohanan, 2002). In addition, Xu et al. (2016) found that melatonin potentials protects against cadmium neurotoxicity by blocking calcium-dependent translocation of Drp1 to the mitochondria. Recently, a study by Sinha et al. (2018) further reported that melatonin can inhibit mitochondrial cell death pathways by upregulating the MT1 receptor in newborn hypoxic-ischemic brain injury mice models. Besides improving the mitochondrial function, melatonin reportedly can act on cerebral nitric oxide/nitric oxide synthase after hypobaric hypoxia injury, which balances the release of nitric oxide, reduces peroxynitrite formation, and protects against nitrosative/oxidative damage (Blanco et al., 2017).

Thus, the collective general findings were that melatonin protects against hypoxemia. Although it is unknown whether melatonin can improve cerebral autoregulation after SAH by ameliorating the reduced brain-tissue oxygen pressure and brain pH, there is a theoretical basis for this hypothesis (**Figure 1**). It is worth mentioning that a study conducted by Herrera et al. (2014) found that, in chronically hypoxic lambs, melatonin improved vascular responses to potassium, serotonin, and methacholine and enhanced the endothelial response via nitric oxide-independent mechanisms in isolated arteries. This study indirectly indicates the possible impact of melatonin on cerebral autoregulation (Herrera et al., 2014).

Comparison of Melatonin With Other Medications in Improving Cerebral Autoregulation

Previous studies have reported that several medications may have the potential to improve cerebral autoregulation after SAH. Nitric oxide plays an important in regulating cerebrovascular tone by maintaining the dilation of the vasculature. After SAH, nitric oxide production and responses to endothelium-dependent vasodilators were impaired owing to injury to the cerebrovascular endothelium, resulting in vasoconstriction (Sobey and Faraci, 1998). Consequently, nitric oxide (or nitric oxide donors) was proposed as a possible medication to improve cerebral autoregulation after SAH (Guo et al., 2016). In contrast to melatonin, nitric oxide improves cerebral autoregulation by activating calcium-dependent potassium channels in vascular smooth muscle, thus maintaining stable vascular tone after SAH.

Vasoactive substances can act on vascular smooth muscle, leading to cerebral arterial vasoconstriction or vasodilation. Some vasoactive substances, such as norepinephrine, adrenomedullin, and indomethacin, may have protective functions in cerebral autoregulation (Armstead et al., 2010a, 2016; Chock et al., 2012). However, no such protective function has been reported for other vasoactive substances, such as sodium nitroprusside (Armstead et al., 2010b; Baerts et al., 2013). It is notable that, unlike that for melatonin, the dose of vasoactive substances should be carefully monitored, as the impact of these drugs on cerebral autoregulation may vary based on the dose.

Additionally, pravastatin was reported has the function to improve cerebral autoregulation after SAH by improving vascular endothelium-dependent relaxation in response to acetylcholine, increasing endothelial nitric oxide synthase activity, and enhancing the vascular protective effects of Olmesartan (Yamamoto et al., 2007). These mechanisms have

similarities and dissimilarities to the mechanisms of the action of melatonin. Additionally, we attempted to identify more antioxidants that have been reported to have effects on cerebral autoregulation, but failed to find any further evidence.

CONCLUSION

Melatonin potentially impacts the control mechanisms of cerebral autoregulation after SAH through antioxidation, protection of endothelial cell integrity, suppression of sympathetic nerve activity, increase in nitric oxide bioavailability, mediation of the myogenic response, and amelioration of hypoxemia. Furthermore, melatonin may have a comprehensive effect on cerebral autoregulation after SAH.

AUTHOR CONTRIBUTIONS

Z-NG, HJ, HS, and YZ wrote the manuscript. JL and HM prepared the figures. XS and YY reviewed and edited the manuscript.

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Chronic Stress Produces Persistent Increases in Plasma Corticosterone, Reductions in Brain and Cardiac Nitric Oxide Production, and Delayed Alterations in Endothelial Function in Young Prehypertensive Rats

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This study was designed to investigate whether oxidative stress, nitric oxide (NO) deficiency and/or endothelial dysfunction (ED) are present in young borderline hypertensive rats (BHR) and whether these pathologies can be causally involved in the initiation of blood pressure (BP) increases. Additionally, we tested the hypothesis that crowding stress, experienced during the peripubertal period, may produce persistent or delayed disorders in corticosterone release, NO synthesis, oxidative status and/or endothelial function that could accelerate BP increases. To test these hypotheses, 5-week-old male BHR and normotensive Wistar-Kyoto rats (WKY) were either kept in control conditions (for 2 and 4 weeks, respectively) or exposed to social stress produced by crowding for 2 weeks (stress). After cessation of crowding, a group of rats of each phenotype was kept in control conditions for the next 2 weeks (post-stress). Systolic BP of 5-week-old BHR was significantly increased vs. age-matched WKY (127 ± 3 vs. 104 ± 3 mmHg, $p < 0.01$) and remained significantly higher throughout the course of the experiment. Despite elevated BP, no signs of oxidative damage to plasma lipids, NO deficiency or ED were observed in control BHR vs. age-matched WKY. Crowding stress elevated plasma corticosterone and accelerated BP increases only in BHR; these effects persisted 2 weeks post-stress. Crowding failed to induce oxidative damage to plasma lipids in either phenotype, but it produced persistent decreases in NO production in the hypothalamus and brainstem of both strains of rats, as well as in the hearts of BHR. In contrast, crowding failed to reduce NO production in the aortae or acetylcholine-induced relaxations of the femoral arteries in both strains investigated. However, significantly reduced aortic NO production was observed in BHR 2 weeks post-stress vs. age-matched controls, which was in agreement with reduced NO-dependent components of vasorelaxation. In conclusion, this study's data showed that oxidative stress, NO deficiency and ED are not causally involved in initiation of blood pressure increase

in BHR. However, exposure to stressful environments produced persistent increases in plasma corticosterone and reductions of brain and cardiac NO production followed by a delayed decrease in the NO-dependent component of endothelium-dependent relaxation—changes that collectively accelerated BP increases only in BHR.

Keywords: crowding stress, borderline hypertension, oxidative stress, corticosterone, nitric oxide, endothelial dysfunction

INTRODUCTION

Hypertension is a widespread disease that increases in prevalence with age (Sun, 2015). However, hypertension or prehypertension (i.e., early stage of hypertension development) are also common among children and adolescents. Prehypertension prevalences of 10–15% were observed among 11- to 17-year-old adolescents (McNiece et al., 2007; Regecova et al., 2011). Even worse, in a study of 4- to 6-year-old children in Spain, the estimates of prehypertension and hypertension prevalence were 12.3 and 18.2%, respectively (Martin-Espinosa et al., 2017). Despite intensive clinical and experimental research, the causes of hypertension remain unknown in approximately 95% of all human cases. Endothelial dysfunction (ED) is a common pathology observed in hypertensive humans (Mordi et al., 2016) and rats (Bernatova, 2014). Oxidative stress and nitric oxide (NO) deficiency frequently cause ED in humans and experimental models of hypertension. Nevertheless, the roles of ED, oxidative stress and NO deficiency in the initiation of hypertension remains unclear (Bernatova et al., 2009; Bernatova, 2014).

Despite persistent discrepancies, chronic stress is widely considered to be an etiological factor associated with the development of cardiovascular diseases (Golbidi et al., 2015), especially when present in early life. Indeed, stressful events in early life increase the risk of elevated blood pressure (BP) in late adulthood (Alastalo et al., 2013). However, there is relatively little information regarding the delayed influence of stress on cardiovascular functioning in young subjects with a family history of hypertension who are genetically predisposed to BP elevation.

Spontaneously hypertensive rats (SHR) are a commonly used rodent model of human primary hypertension (Kunes et al., 2012). Since hypertension development in SHR occurs early in life, it is difficult to investigate metabolic and cardiovascular differences in the pre-hypertensive period. For this purpose, borderline hypertensive rats (BHR) are a more appropriate experimental model of human prehypertension. BHR are produced by mating SHR dams with Wistar-Kyoto (WKY) sires (Fuchs et al., 1998; Slezak et al., 2014). Studies with BHR have shown that this strain is more sensitive to stress than normotensive rats, suggesting a role of stress in the development of high BP in genetically predisposed subjects.

Various rodent experimental models of stress were developed to simulate the effect of stress on BP in order to investigate the mechanisms underlying the development of hypertension (Bouzinova et al., 2014; Crestani, 2016). In our study, we used a model of social stress produced by the crowding that results from reduced living space (Slezak et al., 2014). This model seems to be more relevant to the human condition (D'Atri et al., 1981; Fleming et al., 1987) than other experimental models of chronic stress, as it "typically evokes social stress reactions with prominent psychosocial components, mimicking emotional state alterations" (Bugajski, 1999). Although crowding is a relatively mild stressor, chronic exposure may lead to neuroendocrine, behavioral and cardiovascular alterations (Bugajski et al., 2006; Moiseeva et al., 2009; Bernatova et al., 2010).

This study was designed to investigate whether oxidative stress, NO deficiency and/or ED are present in young BHR males and whether they may be causatively involved in the initiation of BP increases. We also tested the hypothesis that sub-chronic crowding stress, experienced during the peripubertal period, can produce vascular alterations and thus accelerate BP increases in male BHR. Furthermore, we tested the hypothesis that crowding stress may produce persistent or delayed disorders in the hypothalamic-pituitary-adrenocortical (HPA) axis, NO synthesis and/or oxidative status that would produce vascular alterations and/or BP increases.

MATERIALS AND METHODS

Animals and Stress Model

All rats used in our study were born in our animal facility in order to ensure identical conditions for all subjects. BHR were the first filial generation of offspring of SHR dams and WKY sires. Five weeks old male rats were separated from their mothers and randomly allocated into control and crowding stress-exposed groups. Control rats were kept under standard conditions [∼200 cm^2/100 g of body weight (BW)] in groups of four rats per cage for two (C7) and 4 weeks (C9), respectively. Rats exposed to stress were kept in groups of five rats per cage with living space approximately 70 cm^2/100 g of BW for 2 weeks, as described previously (Slezak et al., 2014). Five-week-old WKY ($n = 36$) and BHR ($n = 36$) were housed either in control ($n = 16$, for each phenotype) or crowded conditions ($n = 20$, for each phenotype). After crowding, rats were either killed ($n = 10$, for each phenotype, stress group) or placed back into control conditions ($n = 10$, for each phenotype) for the next 2 weeks (post-stress groups).

All rats were maintained at a temperature 22–24°C and artificial lights (12 h light/dark cycle). The animals were fed a standard pellet diet and tap water *ad libitum*. At the end of the experiment (between 08.00 and 09.00), the rats were decapitated after brief (60–90 s) CO_2 anesthesia.

All experiments were approved by the State Veterinary and Food Administration of the Slovak Republic.

Heart Rate and Blood Pressure

Heart rate (HR) and systolic BP were determined by tail-cuff method between 08.00 and 11.00, as described in detail previously (Puzserova et al., 2013b). Each BP and HR value was calculated as the average of five measurements. BP and HR values were measured at the beginning of the experiment (basal/day 0), and after each week of the experiment (days 7, 14, 21, and 28). BW was determined on the same days. Relative wet mass of the left heart ventricle (LHV) and both adrenal glands (AG) were determined at the end of experiment as the LHV/BW and AG/BW ratio, respectively.

Plasma Corticosterone

Rats were killed by decapitation as described above. Trunk blood samples were collected in heparinized test tubes and immediately centrifuged at 850 g for 10 min at 4°C. Plasma samples were then stored at −80°C until analysis. Plasma corticosterone (pCort) was measured in duplicate in 20 μl of plasma by an enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, United States), as described in detail previously (Puzserova et al., 2013b).

Nitric Oxide Synthase Activity

Activity of NO synthase (NOS) was determined by conversion of [^{3}H]-L-arginine (MP Biomedicals, Santa Ana, CA, United States) in 20% crude tissue homogenates of the LHV, aorta, hypothalamus and brainstem, as described previously (Puzserova et al., 2013b), and was expressed as pmol/min/mg of protein.

Determination of Oxidative Status

Blood was collected into heparin-coated tubes and centrifuged (4°C, 850 g for 10 min) to obtain erythrocytes and plasma. Erythrocytes were washed three times with 150 mmol/l NaCl solution. After next centrifugation (4°C, 900 g for 5 min), erythrocytes were hemolyzed by adding the three volumes of distilled water (4°C). Aliquoted plasma and hemolyzed erythrocytes were stored at −20°C until analyses. Concentration of lipid peroxides (LP) and total antioxidant capacity were determined in plasma. Activities of catalase (CAT), glutathione peroxidase (GPx), Cu/Zn superoxide dismutase (SOD), and concentration of hemoglobin (Hb) (Drabkin and Austin, 1932) were determined in hemolysate of erythrocytes.

Plasma LP level was measured as described previously (el-Saadani et al., 1989) and presented in nmol/ml of plasma. Total antioxidant capacity of plasma was determined according to Re et al. (1999) as the trolox equivalent antioxidant capacity (TEAC) and expressed as mmol of trolox/l. Activities of SOD and GPx were determined using a commercially available kits, Fluka, (Germany) and Enzo Life Sciences (United States), respectively, following the instructions of the manufacturers. SOD activity was expressed in units (U) per mg Hb. GPx activity was expressed in μkat/g Hb. Activity of CAT was determined by the modified method by Bergmeyer (1987) and its activity was expressed in μkat/g Hb.

Vascular Reactivity Measurements

Vascular reactivity of the femoral artery segments (1.0 – 1.5 mm long) was determined using wire myograph (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark). Normalization, including calculations for normalized inner diameter, was performed as described by Puzserova et al. (2013b). The experimental protocol consisted of the following steps: (a) Modified physiological salt solution (PSS, 118.99 mmol/l NaCl, 4.69 mmol/l KCl, 25 mmol/l $NaHCO_3$, 1.17 mmol/l $MgSO_4.7H_2O$, 1.18 mmol/l KH_2PO_4, 2.5 mmol/l $CaCl_2.2H_2O$, 0.03 mmol/l Na_2EDTA, 5.5 mmol/l glucose) in the myograph chamber was changed to depolarizing solution KPSS (i.e., PSS in which NaCl was replaced with KCl with concentration of K^+ 125 mmol/l) for 2 min and contraction was measured. (b) After confirming a sufficient contractile response to KPSS 10 μmol/l of noradrenaline (NA) was added and the phasic and tonic contractions were determined. (c) The segment was pre-constricted with 1 μmol/l 5-hydroxytryptamine (serotonin). Endothelium-dependent relaxation was determined by increasing concentrations of acetylcholine (ACh, from 0.001 to 10 μmol/l), added cumulatively. (d) The same procedure was repeated after pre-incubation (25-min) with 300 μmol/l N^G-nitro-L-arginine methyl ester (L-NAME, non-specific NOS inhibitor). (e) Endothelium-independent relaxation was determined in 5-hydroxytryptamine pre-constricted (1 μmol/l) arteries by cumulative adding of the NO donor sodium nitroprusside (SNP, 0.001 – 10 μmol/l). (f) Finally, PSS was changed again to KPSS to induce maximal contraction and was left to achieve a plateau. The artery was washed out four times with PSS and stabilized for 20 min after each step and 30 min before creating a SNP-induced concentration response curve. Vasorelaxation was expressed as the percentage of the initial steady-state contraction induced by 5-hydroxytryptamine. Endothelium-dependent relaxations were also determined as the area under the curve (AUC, in arbitrary units) calculated under individual concentration-response relaxation curves. NO-mediated relaxation was determined by measuring the portion of ACh-induced relaxation that was inhibited by L-NAME. Vasoconstrictions were determined as the maximal active wall tension (μN/mm).

Concentrations of all substances were expressed as final concentrations in the myograph chamber. All chemicals used in this study were purchased from Merck Chemicals (Germany) and Sigma-Aldrich (Germany), except for noradrenaline hydrogenotartras (Zentiva, Czechia).

Statistical Analysis

Heart rate and blood pressure data were analyzed by three-way ANOVA (phenotype × group × day). Relaxation curves were analyzed by three-way ANOVA (phenotype × group × concentration of ACh). All other data were analyzed by two-way ANOVA (phenotype × group). All analysis were followed by Bonferroni's *post hoc* tests. The significance level of all tests was set at 5% ($\alpha = 0.05$). Correlation between variables was determined using Pearson's correlation coefficient (r). All results are presented as the

mean ± SEM. Data were analyzed with Statistica® 7.0 (Stat Soft, Inc., United States) and GraphPad Prism 5.0 (GraphPad Software, Inc., United States).

RESULTS

Basic Parameters

Blood pressure of 5-week-old WKY and BHR rats differed significantly (104 ± 3 mmHg vs. 127 ± 3 mmHg, $p < 0.01$). There were significant phenotype- and stress-dependent differences in BP and BW of rats, considering the entire duration of the experiment (i.e., fifth to ninth week of rat life). BHR had higher BP (137 ± 2 vs. 111 ± 2 mmHg, $p < 0.0001$, main effect of phenotype) and BW than WKY (180 ± 3 vs. 171 ± 4 g, $p < 0.05$, main effect of phenotype). Stress reduced BW (154 ± 4 vs. 144 ± 3 g, $p < 0.05$, the main effect of stress at the end of stress period).

Interaction analysis (phenotype x stress x time of exposure) revealed that crowding increased BP significantly in BHR vs. control ($p < 0.05$) but not in WKY on day 14. BP of BHR also remained elevated vs. control ($p < 0.05$) 2 weeks after the cessation of crowding—an effect not seen in WKY (**Figures 1A,B**). The HR of 5-week WKY and BHR rats was 456 ± 10 and 454 ± 8 bpm, respectively, and no significant changes were observed during experiments in either phenotype (data not shown).

Seven-week control BHR had significantly higher pCort levels than age-matched WKY (**Figure 1C**). Stress significantly elevated pCort only in BHR vs. control as well as vs. stressed WKY. High levels of pCort persisted in BHR 2 weeks post stress (**Figure 1C**).

There were no phenotype or stress-related differences in the relative weights of the LHV calculated as the LHV weight-to-BW ratio (**Table 1**). Relative weights of the AGs, calculated as the AGs weight-to-BW ratio, were significantly higher in 7-week control BHR vs. WKY. Relative weights of the AGs were unaffected by stress (**Table 1**).

NO Synthase Activity

In the aorta, NOS activity tended to be increased in stressed rats of both phenotypes, resulting in a significant main effect of stress vs. age-matched controls (4.85 ± 0.33 vs. 3.41 ± 0.35 pmol/mg/min, $p < 0.05$, main effect of stress, **Figure 2A**). In the LHV stress reduced NO synthase activity only in BHR (**Figure 2B**). In the hypothalamus and brainstem (**Figures 2C,D**) stress reduced NOS activities in both phenotypes. At the end of post-stress recovery, significantly reduced NOS activities were detected in the all tissues investigated in BHR vs. age-matched controls (**Figures 2A–D**). In WKY, a significant decrease of NOS activity in the hypothalamus and brainstem was observed in the post-stress group compared to age-matched controls (**Figures 2C,D**).

Oxidative Status

No differences in SOD, CAT and GPx activities in erythrocytes as well as in plasma TEAC and LP were found in 7- and 9-week-old control WKY and BHR (**Figures 3A–E**). Stress reduced

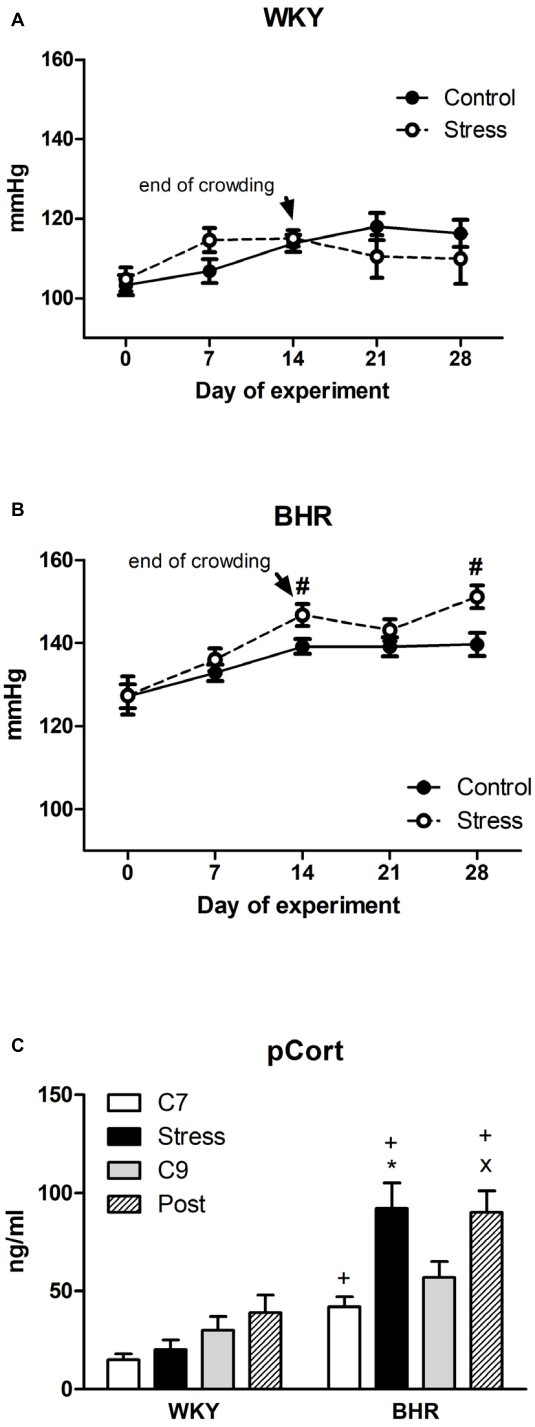

FIGURE 1 | Blood pressure **(A,B)** and plasma corticosterone (pCort, **C**) of normotensive Wistar-Kyoto (WKY) and borderline hypertensive (BHR) rats. Controls (C7 and C9) were kept in control conditions throughout the course of experiment. Stressed rats were exposed to crowding for 2 weeks (days 1–14). After cessation of stress, one group of rats (post-stressed rats, Post) was returned to control conditions for the next 2 weeks. The results are mean ± SEM, n = 8–10/group for blood pressure, n = 6–8/group for pCort. Symbols: $^{\#}p < 0.05$ vs. controls of the same phenotype, $^{*}p < 0.05$ vs. 7-week controls (C7) of the same phenotype, $^{x}p < 0.05$ vs. 9-week controls (C9) of the same phenotype, $^{+}p < 0.05$ vs. the same WKY group.

TABLE 1 | Biometric and vascular parameters of the femoral artery in normotensive Wistar-Kyoto (WKY) and borderline hypertensive rats (BHR).

	WKY				BHR			
	C7 ***n* = 6–8**	**Stress** ***n* = 8–10**	**C9** ***n* = 6–8**	**Post** ***n* = 8–10**	**C7** ***n* = 6–8**	**Stress** ***n* = 8–10**	**C9** ***n* = 6–8**	**Post** ***n* = 8–10**
ND (μm)	649 ± 17	637 ± 16	681 ± 16	691 ± 10$^{\#}$	677 ± 19	659 ± 15	690 ± 11	708 ± 11$^{\#}$
$E_{max\,ACh}$ (%)	87 ± 2	90 ± 2	84 ± 2	84 ± 4	81 ± 3	80 ± 4	83 ± 2	83 ± 3
$E_{max\,SNP}$ (%)	95 ± 1	97 ± 1	97 ± 1	97 ± 1	97 ± 1	99 ± 1	97 ± 1	96 ± 2
NA_p (μN/mm)	518 ± 78	621 ± 48	872 ± 86*	691 ± 174	1253 ± 324^{+}	915 ± 148^{+}	965 ± 252	643 ± 91
NA_t (μN/mm)	71 ± 21	63 ± 11	422 ± 114*	309 ± 105$^{\#}$	1429 ± 639^{+}	771 ± 269^{+}	861 ± 330	1165 ± 549
LHV/BW (mg/g)	1.85 ± 0.05	1.95 ± 0.18	1.56 ± 0.04*	1.63 ± 0.07$^{\#}$	1.82 ± 0.07	1.98 ± 0.06	1.65 ± 0.09	1.81 ± 0.03
AG/BW (mg/100 g)	17.1 ± 0.7	16.1 ± 0.3	13.5 ± 0.3*	13.8 ± 3$^{\#}$	19.5 ± 0.7^{+}	19.3 ± 0.4^{+}	16.4 ± 3*	15.7 ± 0.8$^{\#}$

C7, 7-week controls; C9, 9-week controls; Post, Post-stress; $E_{max\,ACh}$, maximal relaxation to acetylcholine based on individual concentration-response curves; $E_{max\,SNP}$, maximal relaxation to sodium nitroprusside based on individual concentration-response curves; NA_p, maximal phasic response to noradrenaline; NA_t, maximal tonic response to noradrenaline; ND, normalized internal diameter of the femoral artery at 13.3 kPa; SNP, sodium nitroprusside; AG/BW, adrenal glands weight-to-body weight ratio; LHV/BW, left heart ventricle weight-to-body weight ratio. The results are mean ± SEM. $^{*}p < 0.05$ vs. seven-week controls (C7) of the same phenotype, $^{\#}p < 0.05$ vs. stress of the same phenotype, $^{+}p < 0.05$ vs. WKY of the same group.

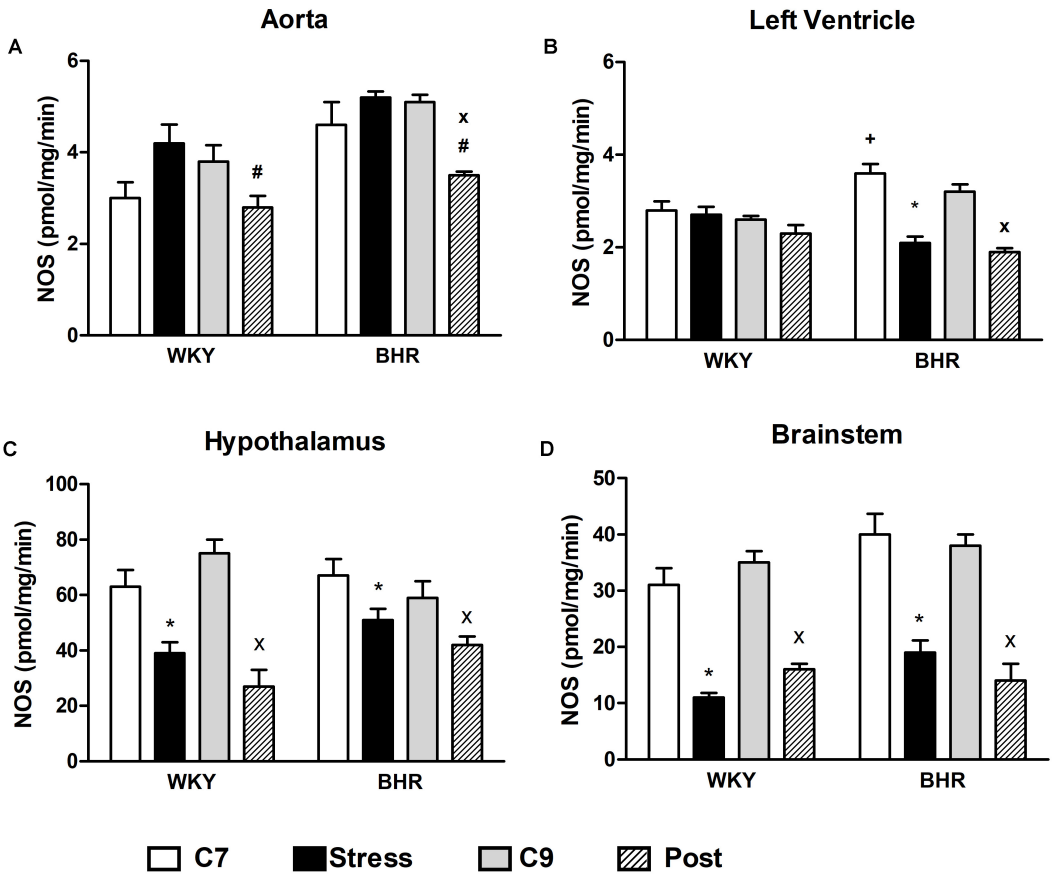

FIGURE 2 | Nitric oxide synthase (NOS) activity in the aorta **(A)**, left heart ventricle **(B)**, hypothalamus **(C)** and brainstem **(D)** of normotensive Wistar-Kyoto (WKY) and borderline hypertensive (BHR) rats in 7-week controls (C7, white bar), stress (black bar), 9-week controls (C9, gray bar) and post-stress (Post, strip bar). In the aorta, the main effect of stress vs. age-matched controls (C7) was significant (see Results section for details). The results are mean ± SEM, n = 6–8/group. Symbols: $^{*}p < 0.05$ vs. 7-week controls (C7) of the same phenotype, $^{\#}p < 0.05$ vs. stress of the same phenotype, $^{x}p < 0.05$ vs. 9-week controls (C9) of the same phenotype, $^{+}p < 0.05$ vs. the same WKY group.

TEAC in BHR compared to stressed WKY (**Figure 3A**). SOD activity was significantly elevated in post-stressed WKY vs. age-matched controls and post-stressed BHR (**Figure 3B**). There was a significant positive correlation between TEAC and LP in BHR ($r = 0.65$, $p < 0.001$, $n = 27$) which was not present in WKY rats ($r = 0.25$, $p = 0.24$, $n = 24$, **Figure 3F**).

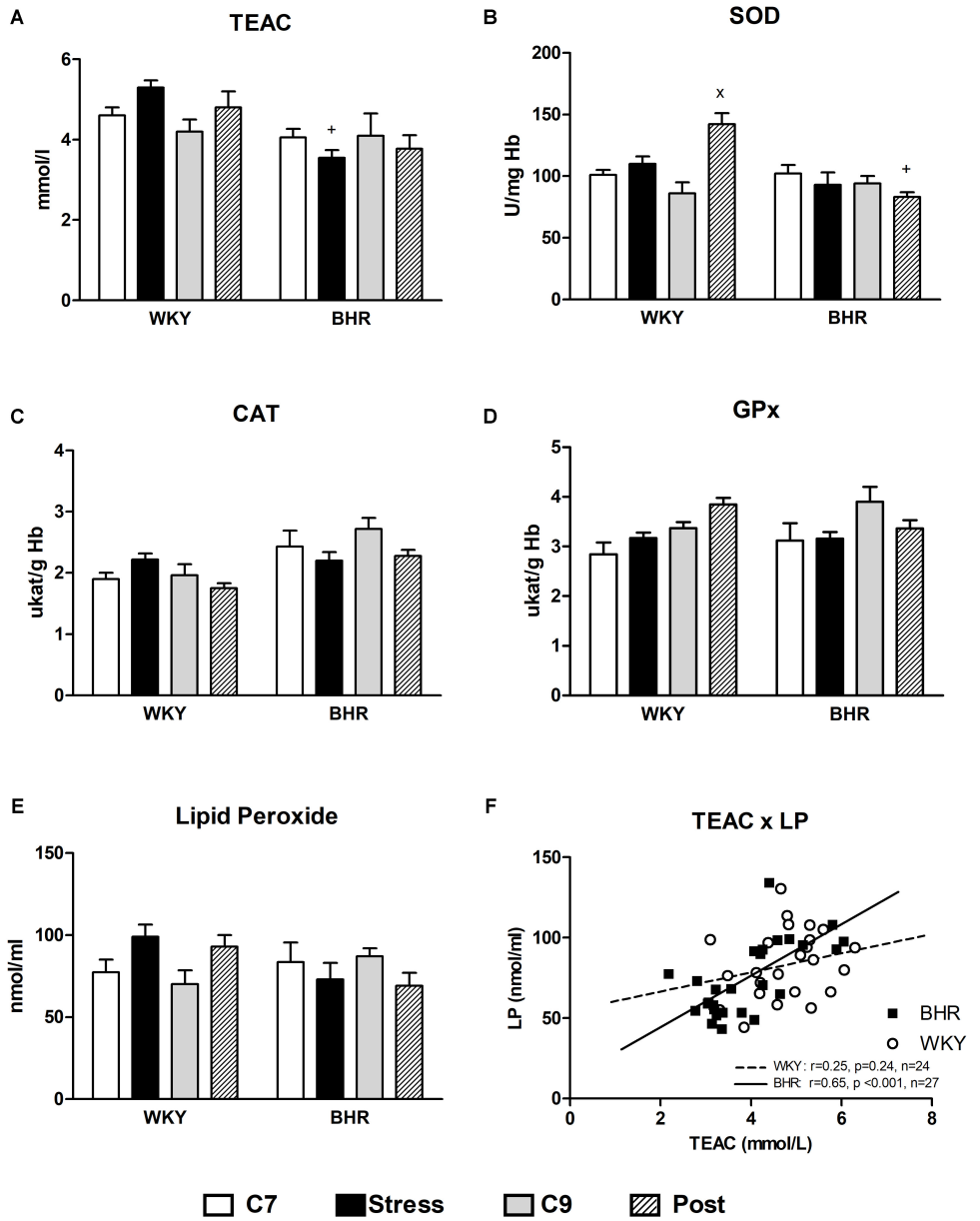

FIGURE 3 | Parameters of oxidative status **(A–E)** in the blood of normotensive Wistar-Kyoto (WKY) and borderline hypertensive (BHR) rats in 7-week controls (C7, white bar), stress (black bar), 9-week controls (C9, gray bar) and post-stress (Post, strip bar). **(F)** Shows correlations between TEAC and LP in WKY and BHR rats. CAT, catalase; GPx, glutathione peroxidase; Hb, hemoglobin; LP, lipid peroxides; SOD, Cu/Zn- superoxide dismutase; TEAC, trolox equivalent antioxidant capacity of plasma. The results are mean ± SEM, n = 6–7/group. Symbols: $^{x}p < 0.05$ vs. nine-week controls (C9) of the same phenotype, $^{+}p < 0.05$ vs. the same WKY group.

Vascular Reactivity

Normalized internal diameter of the femoral artery was similar in age-matched control WKY and BHR. Stress exposure had no effect on this parameter (**Table 1**).

Both endothelium-dependent ACh-induced and endothelium-independent SNP-induced maximal relaxations were similar in all groups investigated (**Table 1**). Five-hydroxytryptamine-induced vasoconstriction was similar among groups (results not shown). NA-induced responses of the femoral artery were biphasic: a transient phasic contraction, which returned nearly to baseline, was followed by sustained tonic contraction. Both NA-induced phasic and tonic responses were elevated in control BHR vs. WKY ($p < 0.03$, main effect of phenotype in all control rats), but this effect was more pronounced in 7-week control rats (**Table 1**). No effects of stress and post-stress recovery on NA-induced vasoconstriction were observed compared to age-matched controls. Similar results for NA contractions were obtained in relative values calculated as

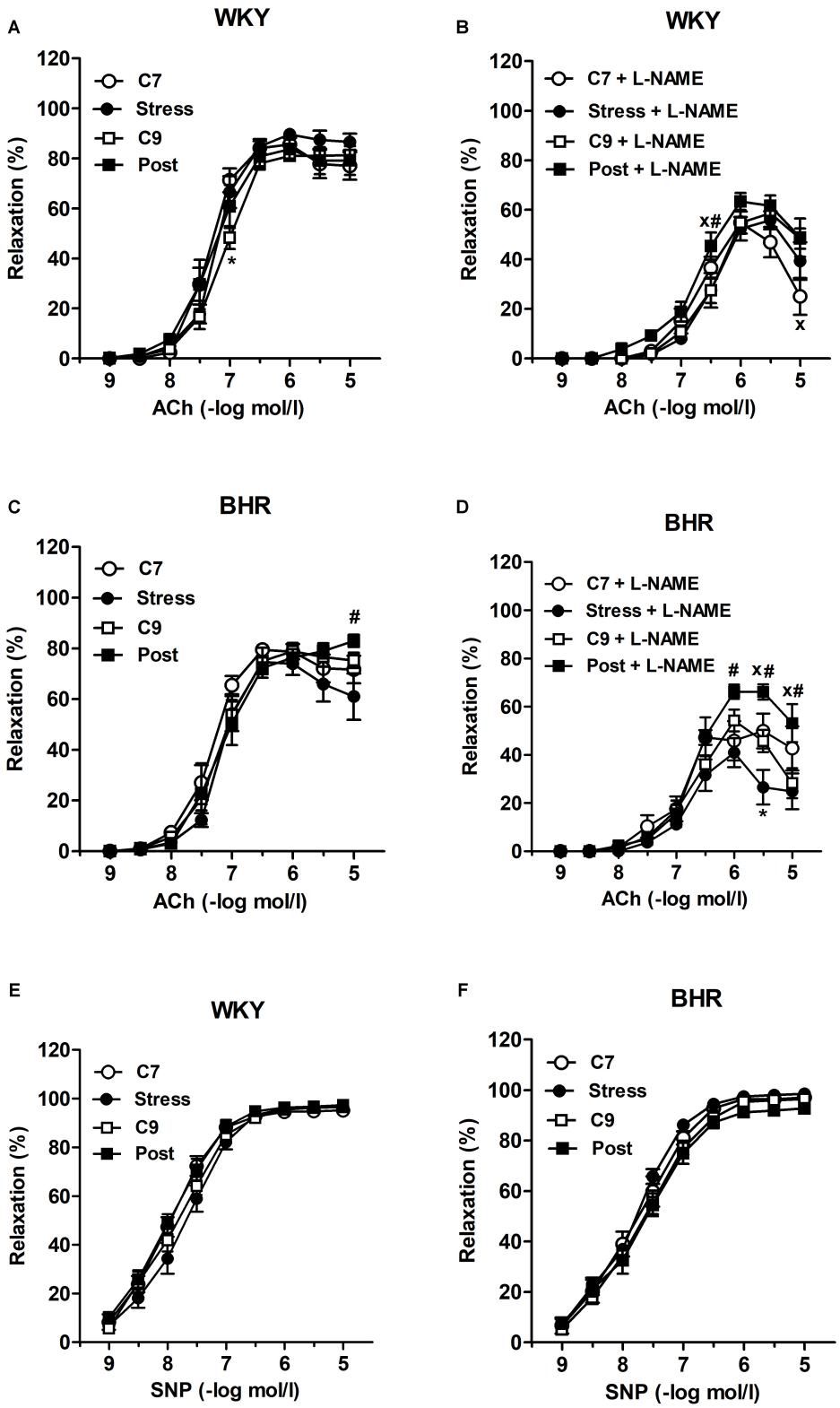

FIGURE 4 | Vascular responses to acetylcholine **(A–D)** and sodium nitroprusside **(E,F)** in the isolated femoral arteries of normotensive Wistar-Kyoto (WKY) and borderline hypertensive (BHR) rats before **(A,C)** and after **(B,D)** incubation with nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μmol/l). ACh, acetylcholine; C7, seven-week controls; C9, nine-week controls; Post, post-stress; SNP, sodium nitroprusside. The results are mean ± SEM, n = 6–10/group. Symbols: $^{*}p < 0.05$ vs. seven-week controls (C7) of the same phenotype, $^{\#}p < 0.05$ vs. stress of the same phenotype, $^{x}p < 0.05$ vs. nine-week controls (C9) of the same phenotype.

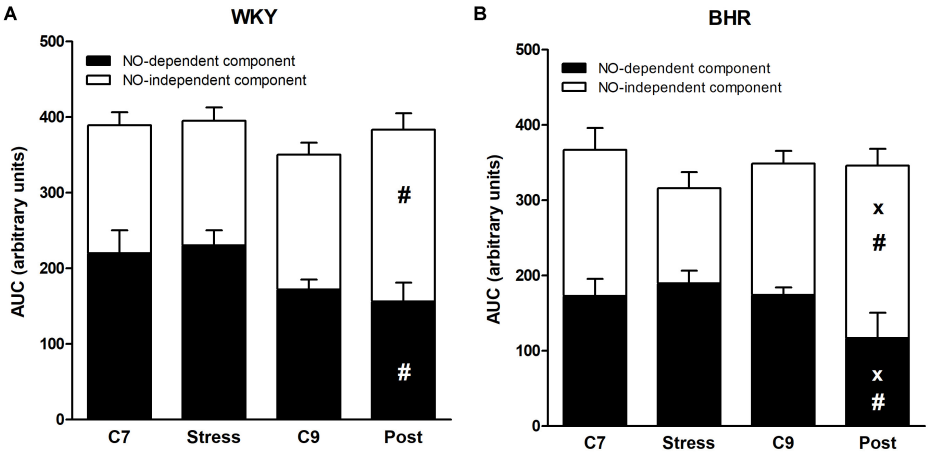

FIGURE 5 | Acetylcholine-induced relaxation of the femoral artery of normotensive Wistar-Kyoto (WKY, **A**) and borderline hypertensive (BHR, **B**) rats. AUC, area under the curve; C7, seven-week controls; C9, nine-week controls; NO, nitric oxide; Post, post-stress. The results are mean ± SEM, n = 6–10/group. Symbols: $^{\#}p < 0.05$ vs. stress of the same phenotype, $^{x}p < 0.05$ vs. nine-week controls (C9) of the same phenotype.

percentage of maximal response induced by KPSS (results not shown).

There were no considerable differences in ACh and SNP-induced concentration-response curves and maximal relaxations among the groups (**Table 1** and **Figures 4A,C,E,F**). Application of L-NAME partially inhibited the ACh-induced relaxation in all groups investigated, as illustrated in **Figures 4B,D**. However, L-NAME attenuated ACh-induced relaxation less markedly in post-stressed BHR than in age-matched controls (**Figure 4D**). Furthermore, the reduced NO-dependent component of ACh-induced relaxation was observed in both post-stressed WKY and BHR vs. the respective stress group, as well as in post-stressed BHR vs. the age-matched control BHR group (**Figures 5A,B**). Conversely, the NO-independent component of vasorelaxation induced by ACh was elevated in the BHR post-stress group vs. the respective age-matched control BHR group, which fully compensated for the decrease of NO-dependent relaxation of the femoral arteries (**Figures 4D**, **5B**).

DISCUSSION

This study showed that 5-week-old male BHR rats—offspring of SHR dams and WKY sires—had significantly elevated BP compared to WKY (i.e., offspring of two normotensive parents). However, there were no differences in activities of enzymes involved in antioxidant defense system, antioxidant capacity of plasma, plasma lipid peroxidation, or endothelium-dependent relaxations of the femoral arteries. Also, there were no signs of reduced NO production in the aorta, LHV, hypothalamus or brainstem in either 7- or 9-week-old control BHR compared to normotensive controls, excluding these pathologies as a cause of BP increase in BHR rats. However, pCort concentrations were elevated in control BHR vs. WKY under restful conditions. In addition, BP and pCort were significantly increased by crowding only in BHR, remaining elevated 2 weeks post-stress, thereby suggesting that neurogenic mechanisms associated with HPA axis perturbance play a role in the initiation of BP rising.

Regarding endothelial function, similar to our above findings, no changes in overall endothelium-dependent relaxation of small mesenteric arteries were found in 15–17-week-old BHR (Fuchs et al., 1998). However, in adult 22-week-old BHR males, in which systolic BP reached ∼140 mmHg, both ED and LHV hypertrophy were determined (Puzserova et al., 2013a), suggesting that ED and LHV hypertrophy in adulthood are the consequences of pressure overload in this genetic model of borderline hypertension (Bernatova, 2014). Furthermore, no signs of reduced NO production in the aorta were observed in this study in the control BHR, in association with unchanged NO-dependent vasorelaxation of the femoral arteries, demonstrating that vascular NO deficiency is not primarily involved in the initiation of BP increases in young BHR. Similarly, no changes in antioxidant defense system enzymes activities or plasma LP concentrations were detected in 7-week-old BHR suggesting that oxidative stress is not involved in the initiation of BP elevation in this strain. However, there are data that suggest an important role of glucocorticoids in the control of BP, as they may increase the pressor response to noradrenaline, angiotensin II and other vasoconstrictors (Puzserova and Bernatova, 2016). In our study, relative weights of the AGs, pCort levels and NA-induced constrictions were higher in 7-week-old control BHR than in WKY rats, which may be involved in the initiation of BP increases.

Exposure to stress produced by crowding was used in our study to investigate whether stress can induce the development of hypertension. We investigated possible involvement of stress in the development of both NO deficiency in the cardiovascular system and ED in young male BHR. Recent studies showed elevated NO production and NO-dependent relaxations in adult normotensive rats exposed to stress (Puzserova et al., 2013b; Bouzinova et al., 2014) in various types of arteries. In this study,

endothelial function and NO-dependent relaxations were similar in young rats of both strains exposed to stress, possibly because of the shorter duration of the stress. Aortic NO production in stressed rats vs. controls was elevated in both strains when calculated as the main effect of stress. In contrast to the findings in the aorta, stress reduced cardiac NO productions in young BHR males in this study. We have shown previously that long-term cardiac NO deficiency results in structural changes such as development of fibrosis and LHV hypertrophy (Babal et al., 1997). Altered cardiac structure, in association with functional changes, can contribute to the maintenance of high blood pressure, even after cessation of the stressor. Indeed, elevated BP and reduced NO production in the LHV were present in BHR 2 weeks post-stress, which was in contrast to WKY rats. In addition, reduced aortic NO production was observed in the aortae 2 weeks post-stress, which was associated with a reduced NO-dependent component of relaxations of the femoral arteries, suggesting a delayed decrease of vascular NO bioavailability in the conduit arteries of BHR. This may result from the long-term glucocorticoid overload, which can produce alterations in the vascular function. For example, exposure to dexamethasone, which is a synthetic glucocorticoid, reduced eNOS expression in the endothelial cell cultures (Wallerath et al., 1999). Simmons et al. (1996) found that dexamethasone reduced iNOS-mediated NO production in the endothelial cells exposed to cytokines, which was associated with reduction in the tetrahydrobiopterin (NOS cofactor) synthesis. In addition, in the isolated mesenteric arteries, dexamethasone reduced nNOS-mediated NO release in the arteries from spontaneously hypertensive but not normotensive rats (Aras-López et al., 2009). Recently, Lee et al. (2015) has revealed that chronic stress induced by restraint resulted in the reduction in the pial artery dilatation that was associated with a significant reduction of nNOS protein expression, without significant changes in eNOS and iNOS expressions. These studies suggest diverse effects of chronic glucocorticoid overload on vascular NO production, which may differ in normotensive and (pre)hypertensive subjects. As this study was not primarily focused on the role of individual NOSs in stress-induced responses, the contributions of individual NOSs in crowding stress-induced NO-dependent vascular changes in the conduit arteries remains to be elucidated.

However, in our study, the decreases in NO-dependent component of vasorelaxation were fully compensated by NO-independent mechanisms, presumably by elevated prostacyclin- and/or endothelium-derived hyperpolarizing factor-mediated relaxations (Bouzinova et al., 2014). We showed that the impairment of overall endothelial function does not contribute to the maintenance of high BP in young, post-stress BHR, despite altered mechanism(s) mediating endothelium-dependent relaxations. However, it is worth noting that reduced NO bioavailability may be implicated in various endocrine/metabolic disorders, independently of BP. Thus, this mechanism may provide a link between the delayed effects of chronic stress and other stress-induced disorders such as atherosclerosis or insulin resistance (Kawashima and Yokoyama, 2004; Kim et al., 2006).

Consistent with the unchanged vasorelaxations, no alterations in oxidative status were observed in stressed rats in this study. However, SOD activities in post-stressed BHR were significantly reduced compared to those in post-stressed WKY. This might be involved in slow progress of oxidative damage to plasma LP in BHR, consistent with the positive correlation between total antioxidant capacity of plasma and LP. A similar correlation was observed previously in rats with various genetic predispositions to hypertension (Horvathova et al., 2016), and may play an important role in hypertension maintenance in later life. In addition, the lack of oxidative damage to lipids in blood does not exclude local tissue oxidative stress. Specifically, oxidative stress in the brain can contribute to hypertension development via increased sympathoexcitation (Kishi and Hirooka, 2012).

We found that crowding led to significant increases in pCort and an acceleration of BP rise only in BHR. Similarly, higher pressor responses to various stressors have been shown previously in adult BHR males compared with WKY (Fuchs et al., 1998; Bechtold et al., 2009; Sarenac et al., 2011). Because crowding stress failed to induce oxidative stress and ED, the differences in crowding-induced BP increases might be related to the central effects of corticosterone. Bechtold et al. (2009) found that endogenous corticosterone acts via hindbrain glucocorticoid receptors to increase the blood pressure responses to stress in adult BHR males, in contrast to WKY.

In this study, crowding stress elevated BP and pCort while reducing NO production in the hypothalamus and brainstem in BHR. NO is an important mediator in regulation of the HPA axis under both rest and stress, that is involved in HPA axis attenuation and activation, respectively (for review see Puzserova and Bernatova, 2016).

Regarding delayed effect of stress, in our study all BP, pCort and NO production in the selected brain areas failed to recover 2 weeks post-stress. Persistent increases of pCort after stress may result from epigenetic mechanisms such as DNA methylation of glucocorticoid receptors (Turecki and Meaney, 2016), which may impede the HPA axis negative feedback regulation. Subsequently, chronic high glucocorticoid levels may decrease NO bioavailability in various brain areas. It has been shown that high levels of glucocorticoids led to alterations in neuronal NO release in the central nervous system, which is an important mechanism in development of hypertension (Goodwin and Geller, 2012).

Thus, the interaction of stress-induced NO insufficiency in the vascular and central nervous system with accentuated noradrenergic function and sympathetic nervous system (SNS) excitation may provoke BP increases in BHR, similar to those in unstressed SHR. In SHR, altered noradrenergic function and increased SNS tone can be considered to be mechanisms underlying hypertension (Hojna et al., 2007; Shinohara et al., 2012; Sterley et al., 2016).

CONCLUSION

This study yields several important results. First, we found that generalized oxidative stress, vascular NO deficiency and ED are not causally involved in the initiation of BP increases in BHR, as these pathologies were not present in 7-week or 9-week control

BHR despite significantly elevated BP vs. young normotensive rats.

Second, we observed that exposure to stressful environments reduced NO production in the hypothalamus and brainstem of WKY without increasing BP. In contrast, the same environments in BHR resulted in persistent increases in pCort, reductions in brain and cardiac NO production, and delayed alterations in endothelial function that worked together to accelerate the increase of BP in BHR. Thus, relatively short-term, mild social stress, experienced in early life, can create persistent impairment in HPA axis regulation followed by accelerations of BP increase in the offspring of hypertensive mothers and normotensive fathers.

AUTHOR CONTRIBUTIONS

IB designed the research studies, conducted selected experiments, performed statistical analysis, and wrote the manuscript. AP and IZ participated in statistical analysis and writing of the manuscript. AP, PB, NS, MH, and ZK carried out the studies and analyzed the data. IB had primary responsibility for the final content. All authors read and approved the final manuscript.

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Ethanol Extract of *Aurantiochytrium mangrovei* 18W-13a Strain Possesses Anti-inflammatory Effects on Murine Macrophage RAW264 Cells

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In this study, the effects of an ethanolic extract of *Aurantiochytrium mangrovei* 18W-13a strain (AM18W-13a) on lipopolysaccharide (LPS)-induced inflammatory responses in RAW264 murine macrophages were studied. Pre-treatment with the AM18W-13a extract significantly suppressed the LPS-induced production of nitric oxide and pro-inflammatory cytokines. RAW264 cells treated with the AM18W-13a extract for 1 and 24 h were subjected to DNA microarray analyses for detecting the differentially expressed genes. The treatment of RAW264 cells with the AM18W-13a extract for 24 h significantly suppressed the expression of several genes associated with inflammation or chemotaxis. Furthermore, treatment with the AM18W-13a extract for 1 h suppressed the expression of *Pde4b*, but induced the expression of *Egr2* and *Egr3* in RAW264 cells. Additionally, the AM18W-13a extract significantly enhanced the expression of certain anti-inflammatory mediators. This study is the first report of the anti-inflammatory effects of the AM18W-13a extract and its mechanism of action in LPS-stimulated murine macrophages.

Keywords: anti-inflammation, *Aurantiochytrium*, microalgae, RAW264 cells, pro-inflammatory cytokines, nitric oxide, lipopolysaccharide

INTRODUCTION

Inflammation is a highly regulated immune response, in which tissues respond to injury and infection for eliminating the cause of injury, repairing damages, and returning to the original healthy state (Morson, 1970). However, inflammatory dysregulation can lead to chronic inflammation, which is now recognized as the cause of a wide range of diseases and disorders, including autoimmune disorders, neurodegenerative disorders, metabolic syndrome, cardiovascular diseases, and even cancers (Medzhitov, 2010; Murakami and Hirano, 2012). The major classes of drugs used to suppress inflammation are non-steroidal anti-inflammatory drugs and corticosteroids. However, their use is limited owing to several undesirable side effects, including peptic ulceration, osteoporosis, high blood pressure, and kidney problems (Sostres et al., 2010). Natural product-derived complementary medicines have recently gained considerable

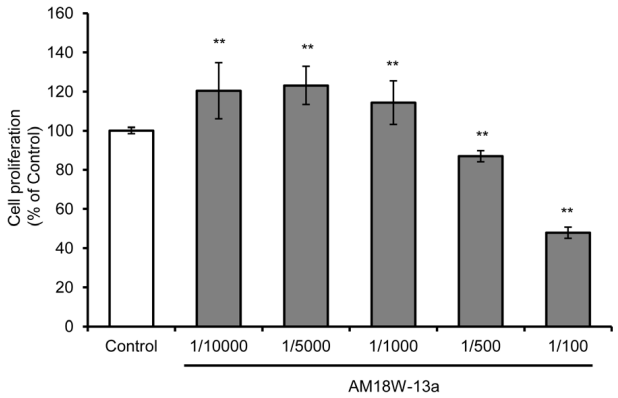

FIGURE 1 | Effects of the AM18W-13a extract on the proliferation of RAW264 cells. The RAW264 cells were treated with the AM18W-13a extract at concentrations of 1/10000, 1/5000, 1/1000, 1/500, and 1/100 for 24 h. The cells in the control group were left untreated. Cell proliferation was measured by the MTT assay after treatment. The values represent the mean ± SD of results obtained from experiments performed in triplicate and are expressed as percentages relative to the results obtained from the control group. The mean value of the experimental result that significantly differed from that of the control group is indicated by asterisks (**$p < 0.01$).

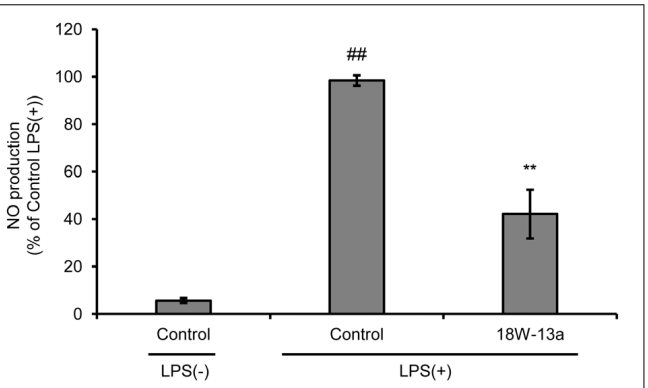

FIGURE 2 | Effects of the AM18W-13a extract (18W-13a) on the LPS-induced production of NO in RAW264 cells. The cells were treated with the AM18W-13a extract at a concentration of 1/1000 for 24 h, while the cells in the control group were left untreated. Following treatment, the cells were activated by incubating with LPS (1 ng/ml) for 12 h. The quantity of NO produced was measured by the Griess reaction. The values represent the mean ± SD of results obtained from experiments performed in triplicate and are expressed as percentages relative to the results obtained from the control LPS (+) group. The mean value of the experimental result that significantly differed from that of the control LPS (–) group is indicated by ##$p < 0.01$. The mean value that is significantly different from that of the control LPS (+) group is indicated by asterisks (**$p < 0.01$).

attention as better alternatives to non-steroidal anti-inflammatory drugs and corticosteroids for treating inflammation, owing to their therapeutic activities. Algae, especially microalgae, are considered to be a promising source of novel bioactive natural compounds that can serve as raw materials for functional foods, cosmetic products, and drug discovery. Several studies have reported the anti-inflammatory effects of microalgal extracts on mammalian cells (Soontornchaiboon et al., 2012; Robertson et al., 2015; Sibi and Rabina, 2016). However, microalgal species produce several other potent and biologically active novel compounds, which require further exploration.

The 18W-13a strain of *Aurantiochytrium mangrovei* (AM18W-13a; previously known as *Aurantiochytrium* sp. 18W-13a) is a microalgal strain, which has been recently discovered and isolated from a mangrove area in the Okinawa Prefecture of Japan (Kaya et al., 2011). It has a very high efficiency of hydrocarbon production, including the production of the hydrocarbon squalene (Nakazawa et al., 2012). The AM18W-13a strain belongs to the genus *Labyrinthula*, which has been reported to contain several bioactive substances, including squalene, astaxanthin, and canthaxanthin (Christaki et al., 2013). It is therefore possible that the AM18W-13a strain is a potential source of numerous biologically active compounds.

At present, one of the potential approaches employed for screening anti-inflammatory drugs involves the inhibition of pro-inflammatory cytokines and the production of nitric oxide (NO) in lipopolysaccharide (LPS)-stimulated macrophages. This study is the first attempt to evaluate the anti-inflammatory activity of an ethanolic extract of the AM18W-13a strain in LPS-stimulated murine RAW264 cells.

MATERIALS AND METHODS

Preparation of the AM18W-13a Extract

The AM18W-13a strain was provided by the Algae Biomass and Energy System R&D Center, University of Tsukuba, Japan. The lyophilized powder of the AM18W-13a strain (0.5 g) was extracted with 5 ml of 99.5% ethanol and kept in the dark at room temperature for 2 weeks. After centrifugation at 190 × *g* for 10 min, the supernatant of the extracted sample was collected and filtered using a 0.22-μm filter unit. The final solution of the extract was then stored in the dark at −80°C until use.

Culture of RAW264 Cells

RAW264 murine macrophages were purchased from RIKEN BioResource Center (RCB0535, RIKEN BRC, Tsukuba, Japan). The RAW264 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin solution at 37°C in a humidified incubator containing 5% CO_2. The cells were seeded in 96-well cell culture plates at a density of 2.0×10^5 cells per well and incubated at 37°C for 24 h.

Preparation of LPS

LPS (*Escherichia coli* serotype O111:B4) was purchased from EMD Millipore Co. (Billerica, MA, United States). LPS (5 mg) was dissolved in 2 ml of phosphate-buffered saline without divalent cations [PBS (−)], and was stored in the dark at −80°C until use.

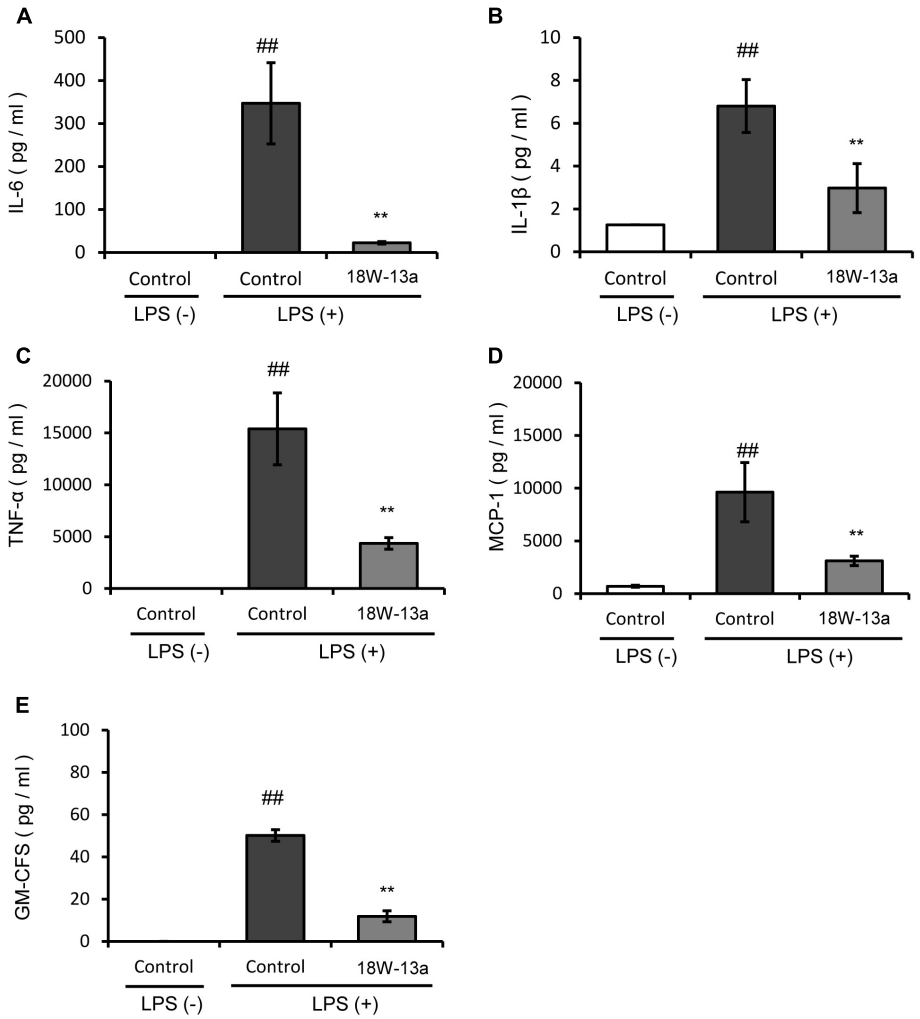

FIGURE 3 | Effects of the AM18W-13a extract (18W-13a) on the LPS-induced expression of pro-inflammatory cytokines in RAW264 cells. The levels of **(A)** IL-6, **(B)** IL-1β, **(C)** TNF-α, **(D)** MCP-1, and **(E)** GM-CSF in RAW264 cells were determined. The cells were treated with the AM18W-13a extract at a concentration of 1/1000 for 24 h, while the cells in the control group were left untreated. Following treatment, the cells were activated by incubation with LPS (1 ng/ml) for 12 h. The pro-inflammatory mediators secreted thereafter were determined by ELISA with the MAGPIX xPONENT system. The values represent the mean ± SD of results obtained from experiments performed in duplicate. The mean value of the experimental result that significantly differed from that of the control LPS (–) group is indicated by $^{\#\#}p < 0.01$. The mean value of the experimental result that significantly differed from that of the control LPS (+) group is indicated by $^{**}p < 0.01$.

Cell Proliferation Assay

The effects of the AM18W-13a extract on the proliferation of RAW264 cells were determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan (Mosmann, 1983). The RAW264 cells were treated with the AM18W-13a extract at concentrations ranging from 1/10000 to 1/100 at 37°C for 24 h, while the cells in the control group were left untreated. Following treatment, the MTT solution was added to each well (10 μl/well) and incubated at 37°C for 4 h. The formazan crystals thus formed were dissolved by the addition of 100 μl of 10% sodium dodecyl sulfate (Wako, Japan) to each well, and incubated overnight at 37°C. After incubation, the absorbance was measured at 570 nm using a microplate reader (Power Scan HT, BioTek Japan Inc.). The absorbance values were normalized to those of the medium and are represented as a percentage of the control (medium).

Measurement of NO Production

The total concentration of nitrite, which is the metabolic end product of NO metabolism, was measured using the two-step Griess diazotization reaction (Sharma et al., 2007). The RAW264 cells were treated with the AM18W-13a extract at 37°C for 24 h. LPS solution (1 ng/ml) was subsequently added to each well and incubated at 37°C for 12 h. The cell culture supernatant was then mixed with an equal volume of Griess reagent [1% sulfanilic acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid; 1:1]. The absorbance was measured at 540 nm using a microplate reader (Power Scan HT, BioTek Japan Inc.). The concentration

TABLE 1 | Gene clusters whose expression was more than 1.4 times higher or lower than that in the control group, following treatment with the AM18W-13a extract for 1 h.

Upregulated genes			
Gene symbol	**Gene name**	**Ratio**	
		1 h	**24 h**
Vapb	Vesicle-associated membrane protein, associated protein B and C	1.88	1.28
Rgs1	Regulator of G-protein signaling 1	1.70	0.51
Egr3	Early growth response 3	1.53	1.09
Naa35	N(alpha)-acetyltransferase 35, NatC auxiliary subunit	1.56	0.90
Egr2	Early growth response 2	1.55	1.05
Zfp146	Zinc finger protein 146	1.49	1.02
Downregulated genes			
Gene symbol	**Gene title**	**Ratio**	
		1 h	**24 h**
Ndufs8	NADH dehydrogenase (ubiquinone) Fe-S protein 8	0.51	1.02
Cxcl2	Chemokine (C-X-C motif) ligand 2	0.52	0.58
Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, ze	0.60	1.12
Ppm1b	Protein phosphatase 1B, magnesium dependent, beta isoform	0.61	0.87
Hnrnpab	Heterogeneous nuclear ribonucleoprotein A/B	0.62	1.29
Pde4b	Phosphodiesterase 4B, cAMP specific	0.66	1.15
Traf1	TNF receptor-associated factor 1	0.68	0.94
Nfkbie	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, ep	0.69	1.04
Ier3	Immediate early response 3	0.70	0.64

The values indicate the average of results obtained from independent experiments performed in duplicate or triplicate.

of NO was calculated from the standard curve of sodium nitrite.

Measurement of Inflammatory Cytokines

The cells were treated with the AM18W-13a extract at 37°C for 24 h. LPS solution (1 ng/ml) was subsequently added to each well, and incubated at 37°C for 12 h. The levels of inflammatory cytokines in the cell culture supernatant were measured using the Bio-Plex Pro™ Mouse Cytokine assay kits (Bio-Rad, United States) according to the manufacturer's instructions. The data were obtained using the MAGPIX xPONENT 4.2 system (Merck Millipore Co., United States). Specific antibodies against tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, monocyte chemoattractant protein-1 (MCP-1), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were used for this assay.

DNA Microarray Analysis for Gene Expression Profiling in RAW264 Cells

The RAW264 cells were seeded in 6-well plates at the density of 2.0×10^5 cells/well and incubated at 37°C for 24 h. The cells were subsequently treated with the AM18W-13a extract at 37°C for 1 and 24 h. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. The amplified RNA (aRNA) was synthesized using the GeneChip 3′ IVT PLUS Reagent Kit (Thermo Fisher Scientific, United States). Hybridization was achieved using the Affymetrix GeneChip Mouse Genome 430 Array Strip (Thermo Fisher Scientific). The images were obtained by the GeneAtlas™ Imaging Station and analyzed using the GeneAtlas™ Workstation (Thermo Fisher Scientific), according to the manufacturer's instructions.

The DNA microarrays were classified and analyzed by the gene set enrichment analysis approach, using the Database for Annotation, Visualization and Integrated Discovery (DAVID) server, v6.8 (National Institute of Allergy and Infectious Diseases (NIAID), NIH, United States[1]) (Huang et al., 2007a,b). The genes containing the gene ontologies for inflammatory response, immune response, chemotaxis, and other cellular activities such as cytokine expression, NO signaling, response to LPS, response to wounding, nuclear factor kappa (NFk) B signaling, and Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling were manually selected from the clustered genes.

Analysis of the Gene Expression of RAW264 Cells Using Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The effects of the AM18W-13a extract on the gene expression of RAW264 cells were evaluated using real-time RT-PCR. The RAW264 cells were seeded in 6-well plates at a density of 2.0×10^5 cells/well and incubated at 37°C for 24 h. RAW264 cells were treated with the AM18W-13a extract at 37°C for 3 h. Total RNA was isolated using ISOGEN (Nippon Gene), according to the manufacturer's instructions. The complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the SuperScript III reverse transcription kit, according to the manufacturer's protocol.

TaqMan real-time RT-PCR amplification reactions were carried out for quantifying the transcripts using the Applied Biosystems 7500 Fast Real-Time System (Thermo Fisher Scientific). The primer sets and the TaqMan Universal PCR Master Mix were obtained from Thermo Fisher Scientific. Specific primers for *Gapdh* (Mm99999915_g1), *Atf3* (Mm00476033_m1), *Socs1* (Mm00782550_s1), and *Socs3* (Mm00545913_s1) were used.

Statistical Analyses

All the results represent the mean of three replicate determinations ± standard deviation (SD). Student's *t*-test

[1]https://david.ncifcrf.gov/

was used for statistical evaluation. Statistical significance was considered at $p < 0.05$, unless otherwise stated.

RESULTS

Effects of the AM18W-13a Extract on the Proliferation of RAW264 Cells

The results of the MTT assay demonstrated that treatment with the AM18W-13a extract at concentrations ranging from 1/10000 to 1/1000 significantly increased the proliferation of RAW264 cells by 20%, in comparison to that of the untreated cells ($p < 0.01$). However, treatment with the AM18W-13a extract at concentrations of 1/500 and 1/100 significantly reduced the proliferation of RAW264 cells to 80 and 50%, respectively (**Figure 1**). Therefore, the AM18W-13a extract was used at a dilution of 1/1000 for further experiments.

Effects of the AM18W-13a Extract on the Proliferation and Production of NO in LPS-Stimulated RAW264 Cells

We evaluated the effects of LPS on the proliferation of RAW264 cells and the production of NO. We found that the proliferation of LPS (0.01–100 ng/ml)-treated RAW264 cells was significantly reduced (**Supplementary Figure S1**), while the production of NO was significantly enhanced in a dose-dependent manner, following treatment with the extract (**Supplementary Figure S2**). The peak of NO production was observed when the concentration of LPS was 1 ng/ml. The cells were therefore treated with 1 ng/ml LPS in the subsequent experiments performed. Treatment with the AM18W-13a extract at a concentration of 1/1000 for 24 h significantly reduced the LPS-induced production of NO ($p < 0.01$) by approximately 50% (**Figure 2**).

Effect of the AM18W-13a Extract on the LPS-Induced Production of Pro-Inflammatory Cytokines

The LPS-induced production of cytokines in the RAW264 cells treated with the AM18W-13a extract was compared to that of the untreated cells. **Figure 3** demonstrates that the levels of IL-6, IL-1β, TNF-α, MCP-1, and GM-CSF in the cells treated with the AM18W-13a extract were significantly lower than those of the untreated cells ($p < 0.01$).

TABLE 2 | Genes whose expression was more than 1.5 times lower than that in the control group, following treatment with the AM18W-13a extract for 24 h, were selected from the gene clusters.

Gene symbol	Gene name	Ratio	
		1 h	24 h
Trem3	Triggering receptor expressed on myeloid cells 3	0.97	0.57
Tnf	Tumor necrosis factor	0.68	0.56
Abcc1	ATP-binding cassette, subfamily C (CFTR/MRP), member 1	1.08	0.54
Flt1	FMS-like tyrosine kinase 1	1.03	0.54
Gch1	GTP cyclohydrolase 1	1.02	0.54
Pdgfb	Platelet derived growth factor, B polypeptide	1.12	0.53
Plau	Plasminogen activator, urokinase	0.99	0.53
S100a8	S100 calcium binding protein A8 (calgranulin A)	1.06	0.53
Prkar2b	Protein kinase, cAMP dependent regulatory, type II beta	1.02	0.52
Slpi	Secretory leukocyte peptidase inhibitor	1.01	0.52
Irg1	Immunoresponsive gene 1	0.94	0.48
Serpinb9	Serine (or cysteine) peptidase inhibitor, clade B, member 9	1.03	0.47
Slc11a1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member	0.90	0.47
Dcstamp	Dendrocyte expressed seven transmembrane protein	1.05	0.46
Gstp1	Glutathione S-transferase, pi 1	1.01	0.46
Nrp1	Neuropilin 1	0.98	0.45
Ptgir	Prostaglandin I receptor (IP)	0.91	0.44
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	1.14	0.44
Ccl4	Chemokine (C-C motif) ligand 4	1.12	0.43
Clec4n	C-type lectin domain family 4, member n	0.92	0.41
Cd36	CD36 antigen	0.98	0.41
Ptgs2	Prostaglandin-endoperoxide synthase 2	1.12	0.39
Ccl3	Chemokine (C-C motif) ligand 3	1.12	0.34
Hmox1	Heme oxygenase (decycling) 1	1.34	0.31

The values indicate the average of results obtained from independent experiments performed in duplicate or triplicate.

Analysis of Gene Expression in RAW264 Cells Treated With the AM18W-13a Extract

The anti-inflammatory properties and the target genes of the AM18W-13a extract were determined by gene expression profiling using DNA microarray analysis of the RAW264 macrophages treated with the AM18W-13a extract. Three independent biological replicates were selected for each condition in the DNA microarray analysis. The differentially expressed genes were analyzed by gene-enrichment analysis, using the web-based tool, DAVID. One hour after treatment with the AM18W-13a extract at a concentration of 1/1000, the expression of six genes was upregulated, while that of nine genes was downregulated (fold change >1.4 and <1.4; **Table 1**). The expression of genes encoding transcriptional regulators and zinc-finger proteins were significantly affected (**Supplementary Table S1**). In particular, the expression of genes encoding the zinc finger transcription factors, such as early growth response 2 (*Egr2)* and *Egr3*, increased following treatment with the extract, while the expression of phosphodiesterase 4B (*Pde4b*) decreased after treatment with the AM18W-13a extract.

Treatment with the AM18W-13a extract at a concentration of 1/1000 for 24 h upregulated the expression of 102 genes and downregulated the expression of 88 genes (fold change >1.5 and <1.5; **Supplementary Tables S2**, **S3**). We used the DAVID gene ontology tool for identifying the genes related to inflammatory responses in the RAW264 cells that had been treated with the AM18W-13a extract. The genes were classified into 35 functionally annotated clusters, out of which six were selected that contained the following terms: chemotaxis, inflammatory, immune response, cytokine-cytokine receptor interaction, and chemokine activity. Finally, 24 genes with downregulated expression profiles and 22 genes with upregulated expression profiles were selected (**Tables 2**, **3** and **Supplementary Tables S4**, **S5**). The expression of genes encoding inflammatory substances, including C-C motif chemokine ligand 3 (*Ccl3*), *Ccl4*, *Tnf*, and prostaglandin-endoperoxide synthase 2 (*Ptgs2*), decreased after treatment with the AM18W-13a extract. Additionally, the expression of genes encoding proteins involved in inflammatory responses or chemotaxis, including ATP-binding cassette subfamily c member 1 (*Abcc1*), cluster of differentiation 36 (*Cd36*), fms-related tyrosine kinase 1 (*Flt1*), S100 calcium binding protein a8 (*S100a8*), and serpin family E member 1 (*Serpine1*) decreased following treatment with the extract (**Table 2** and **Supplementary Table S4**). However, the expression of certain genes involved in the anti-inflammatory pathways, including sphingosine-1-phosphate receptor 1 (*S1pr1*), IL 10 receptor subunit alpha (*IL10ra*), and TSC22 domain family member 3 (*Tsc22d3*), increased after treatment with the AM18W-13a extract (**Table 3** and **Supplementary Table S5**).

TABLE 3 | Genes whose expression was 1.5 times higher than that in the control group, following treatment with the AM18W-13a extract for 24 h, were selected from the gene clusters.

Gene symbol	Gene name	Ratio	
		1 h	24 h
St6gal1	Beta galactoside alpha 2,6 sialyltransferase 1	1.02	2.35
S1pr1	Sphingosine-1-phosphate receptor 1	1.02	2.12
Fcgr3	Fc receptor, IgG, low affinity III	0.90	2.07
Lgals9	Lectin, galactose binding, soluble 9	1.02	2.03
Clec7a	C-type lectin domain family 7, member a	1.02	2.00
Cxcr4	Chemokine (C-X-C motif) receptor 4	0.95	1.99
Blnk	B cell linker	0.99	1.87
Cd28	CD28 antigen	0.93	1.86
Tgfbr1	Transforming growth factor, beta receptor I	1.08	1.84
Ifi44l	Interferon-induced protein 44	1.00	1.82
Il10ra	Interleukin 10 receptor, alpha	0.79	1.78
Lyst	Lysosomal trafficking regulator	0.97	1.77
Tifab	TRAF-interacting protein with forkhead-associated domain, family member B	0.97	1.76
Tsc22d3	TSC22 domain family, member 3	1.02	1.75
Gas6	Growth arrest specific 6	0.93	1.74
Cxcl16	Chemokine (C-X-C motif) ligand 16	1.00	1.72
C3ar1	Complement component 3a receptor 1	1.00	1.70
Aif1	Allograft inflammatory factor 1	0.92	1.66
Irf9	Interferon regulatory factor 9	1.03	1.63
C1qb	Complement component 1, q subcomponent, beta polypeptide	1.03	1.60
Il18	Interleukin 18	1.01	1.52
Il6ra	Interleukin 6 receptor, alpha	1.07	1.56

The values indicate the average of results obtained from independent experiments performed in duplicate or triplicate.

Effects of the AM18W-13a Extract on the Expression of Anti-inflammatory Mediators

The expression of certain genes that negatively regulate the inflammatory process in the RAW264 cells treated with the AM18W-13a extract was evaluated. Treatment with the AM18W-13a extract at a concentration of 1/1000 for 3 h significantly increased the expression of *Socs3* ($p < 0.05$) and *Atf3* ($p < 0.01$) by 4.41- and 1.84-fold, respectively, compared to the control group. Additionally, the expression of *Socs1* tended to increase after treatment with the AM18W-13a extract (**Figure 4**).

The interactions of the anti-inflammatory regulators and the two zinc finger-type transcription factors, *Egr2* and *Egr3*, were investigated using the GeneMANIA server[2], which can predict several gene interaction networks from a gene list (Warde-Farley et al., 2010). Among *Atf3*, *Egr2*, *Egr3*, *Socs1,* and *Socs3*, the coexpression of *Egr2* with *Egr3*, *Atf3*, and *Socs3* and the coexpression of *Egr3* with *Egr2*, *Atf3*, and *Socs1* were confirmed (**Supplementary Figure S3**).

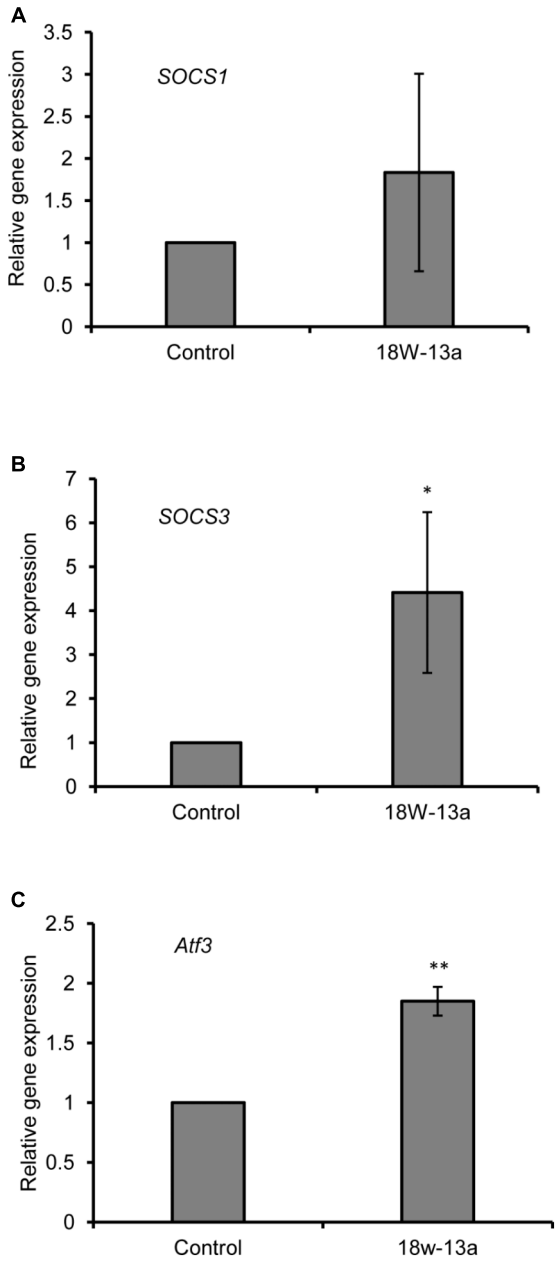

FIGURE 4 | Effects of the AM18W-13a extract (18W-13a) on the expression of *Socs1, Socs3,* and *Atf3* genes in RAW264 cells. The RAW264 cells were treated with the AM18W-13a extract at a concentration of 1/1000 for 3 h, while the cells in the control group were left untreated. Following treatment, the expression of **(A)** *Socs1,* **(B)** *Socs3*, and **(C)** *Atf3* was evaluated by real-time RT-PCR. The values represent the mean ± SD of results obtained from experiments performed in triplicate. The mean value of the experimental result that significantly differed from those of the control group is indicated by asterisks (*$p < 0.05$ and **$p < 0.01$).

DISCUSSION

In this study, we evaluated the anti-inflammatory effects of the AM18W-13a extract on LPS-induced responses in murine RAW264 cells. Macrophages play key roles in all stages of the inflammatory response by acquiring distinct functional phenotypes that are directed by the tissue type and environmental cues (Mosser and Edwards, 2008). LPS is a potent activator of macrophages that triggers the abundant secretion of pro-inflammatory cytokines and the production of NO, which is an inflammatory mediator. The results of this study indicated that the AM18W-13a extract could potentially inhibit the LPS-induced production of NO and the secretion of pro-inflammatory cytokines, including IL-6, IL-1β, TNF-α, MCP-1, and GM-CFS, in RAW264 cells. Moreover, the expression of certain inflammatory substances, including *Ccl3*, *Ccl4*, *Tnf,* and *Ptgs2*, was substantially attenuated by the AM18W-13a extract in LPS-stimulated macrophages. Since these genes were related to 'chemotaxis' and 'inflammation,' it can be assumed that the AM18W-13a extract had anti-inflammatory effects on RAW264 cells.

The treatment of RAW264 cells with the AM18W-13a extract significantly enhanced the expression of *Socs3* and *Atf3*. The expression of *Socs1* also tended to increase following treatment with the extract. The majority of SOCS proteins act in a classical negative-feedback loop for inhibiting cytokine signal transduction. Both the SOCS1 and SOCS3 proteins directly inhibit the tyrosine kinase activity of JAKs and act as negative regulators of the cytokine-induced JAK/STAT pathway. It has also been suggested that SOCS proteins are important negative regulators of toll-like receptor (TLR) signaling. SOCS3 inhibits the IL-1-induced transcription and activation of signaling pathways mediated by NF-κB and JNK/p38 by inhibiting the association between TNF receptor-associated factor 6 and TGF β-activated kinase 1 (Frobose et al., 2006). Additionally, SOCS3 strongly inhibits the IL-6-induced activation of STAT3 but does

[2]http://genemania.org

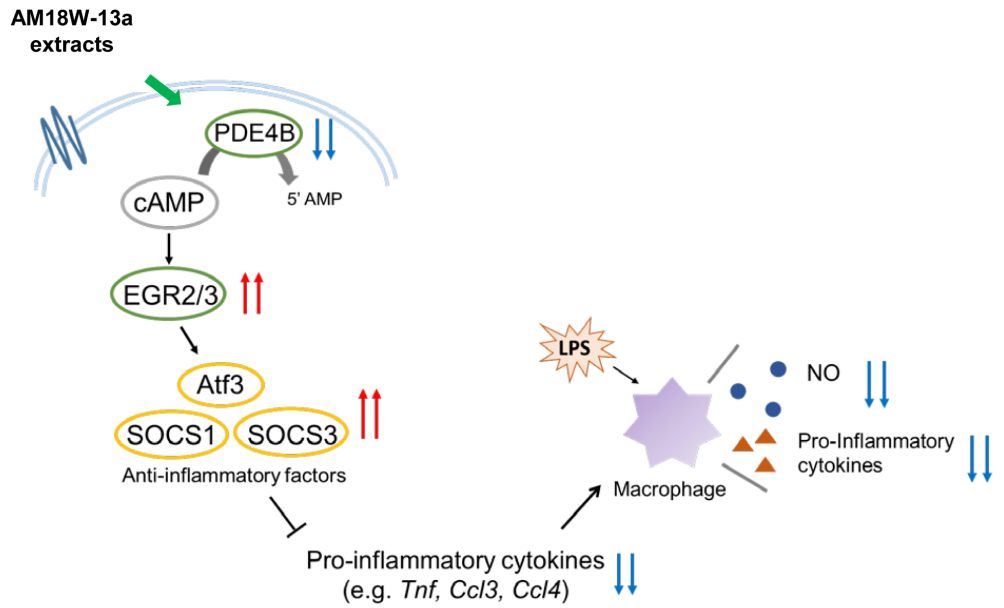

FIGURE 5 | Prediction of the anti-inflammatory effects of the AM18W-13a extract in RAW264 cells. The AM18W-13a extract inhibits the production of LPS-induced inflammatory mediators by increasing the expression of anti-inflammatory mediators such as ATF3, SOCS1, and SOCS3.

not affect the IL-10-induced activation of STAT3. Therefore, the sustained activation of STAT3 inhibits the TLR-induced production of cytokines (Yoshimura et al., 2012). However, SOCS1 binds to the p65 subunit of NF-κB and induces its degradation, thus subsequently inhibiting the NF-κB pathway (Strebovsky et al., 2011). The lack of SOCS1 in macrophages makes them hypersensitive to stimuli, such as LPS, resulting in the increased production of pro-inflammatory cytokines (Strebovsky et al., 2011). ATF3 is a member of the ATF/cyclic adenosine monophosphate (cAMP) response element-binding protein of the basic leucine zipper transcription factors. ATF is reported to be an inducible negative regulator of TLR4 signaling (Gilchrist et al., 2006). A previous study reported that the expression of genes encoding pro-inflammatory cytokines (IL-6 and IL-12b) increases in *Atf3*-deficient mice, indicating that ATF3 negatively regulates the expression of genes related to the inflammatory response (Gilchrist et al., 2008). Additionally, the expression of pro-inflammatory cytokines is significantly reduced in the macrophages of congenic $Atf3^{+/+}$ mice under both LPS-stimulated and unstimulated conditions (Khuu et al., 2007). ATF3 also inhibits the NF-κB signaling pathway by binding either to the promoter of the NF-κB-dependent genes (Gilchrist et al., 2006) or by directly binding to the p65 subunit of NF-κB (Kwon et al., 2015). Altogether, our findings suggest that the anti-inflammatory effects of the AM18W-13a extract could be related to the increased expression of *Atf3*, *Socs1,* and *Socs3*.

Furthermore, the results of DNA microarray analysis revealed that treatment with the AM18W-13a extract for 1 h suppressed the expression of *Pde4b* and enhanced the expression of *Egr2* and *Egr3* in RAW264 cells. PDE4B is a member of the PDE enzyme superfamily, which hydrolyzes cAMP to the inactive 5′ monophosphatase. Since cAMP exhibits anti-inflammatory properties in a variety of cell types (Oldenburger et al., 2012), the degradation of cAMP by PDE promotes the pathogenesis of inflammatory diseases. *Egr2* and *Egr3* are well-known homeostasis-related genes that encode proteins of the EGR family, which are zinc finger transcription factors. EGRs play an important role in the development of systemic autoimmune diseases, and the deficiency of EGR2 and EGR3, in particular, are related to the development of chronic inflammatory diseases, such as lupus, in murine models (Gomez-Martin et al., 2010). Recent studies have demonstrated that EGR2 and EGR3 induce the expression of anti-inflammatory regulators, including SOCS1 and SOCS3 (Li et al., 2012; Sumitomo et al., 2013). Additionally, lymphocytes deficient in *Egr2* and *Egr3* have been reported to produce high levels of inflammatory cytokines (Li et al., 2012). Interestingly, the expression of EGR2 is induced when the intracellular levels of cAMP are elevated (Schmid et al., 2014). We can therefore hypothesize that the AM18W-13a extract inhibits the intracellular degradation of cAMP by regulating the expression of the *Pde4b* gene, and the resulting increase in levels of intracellular cAMP induces the expression of *Egr2* and *Egr3*, which subsequently enhances the expression of *Socs1* and *Socs3*. This mechanism of post-transcriptional regulation has not been proven by direct experimental evidence, but by network-level conservation for confirming the coexpression of *Atf3*, *Egr2*, *Egr3*, *Socs1,* and *Socs3*. It was found that *Egr2* is coexpressed with *Egr3*, *Atf3*, and *Socs3*, while *Egr3* is coexpressed with *Egr2*, *Atf3*, and *Socs1*.

In summary, the study reports that the AM18W-13a extract may inhibit the production of NO and the secretion of pro-inflammatory cytokines by upregulating the expression of anti-inflammatory mediators, including ATF3, SOCS1, and SOCS3,

by inducing the expression of the transcription factors EGR2 and EGR3, via cAMP (**Figure 5**). These results indicate that the AM18W-13a extract has anti-inflammatory effects on murine macrophages.

AUTHOR CONTRIBUTIONS

ST, MS, and HI conceived and designed the experiments. MS performed the experiments. ST, MS, and FF prepared the figures, tables, and manuscript. ST and MS analyzed and interpreted the results. MY, MD, and MW provided the AM18W-13a strain. HI edited and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01205/full#supplementary-material

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REVIEW
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Implication of Oxidative Stress in Fetal Programming of Cardiovascular Disease

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Lifestyle and genetic background are well known risk factors of cardiovascular disease (CVD). A third contributing factor is suboptimal fetal development, due to nutrient or oxygen deprivation, placental insufficiency, or exposure to toxic substances. The fetus adapts to adverse intrauterine conditions to ensure survival; the immediate consequence is low birth weight (LBW) and the long-term effect is an increased susceptibility to develop CVD in adult life. This process is known as Developmental Origins of Health and Disease (DOHaD) or fetal programming of CVD. The influence of fetal life for the future cardiovascular health of the individual has been evidenced by numerous epidemiologic studies in populations suffering from starvation during intrauterine life. Furthermore, experimental animal models have provided support and enabled exploring the underlying mechanisms. Oxidative stress seems to play a central role in fetal programming of CVD, both in the response of the feto-placental unit to the suboptimal intrauterine environment and in the alterations of physiologic systems of cardiovascular control, ultimately leading to disease. This review aims to summarize current knowledge on the alterations in oxidative balance in response to fetal stress factors covering two aspects. Firstly, the evidence from human studies of the implication of oxidative stress in LBW induced by suboptimal conditions during intrauterine life, emphasizing the role of the placenta. In the second part we summarize data on specific redox alterations in key cardiovascular control organs induced by exposure to known stress factors in experimental animals and discuss the emerging role of the mitochondria.

Keywords: oxidative stress, fetal programming, cardiovascular diseases, fetal growth restriction (FGR), mitochondrial dysfunction

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FETAL PROGRAMMING OF CARDIOVASCULAR DISEASES

The Developmental Origins Hypothesis

Cardiovascular diseases (CVD) are one of the leading causes of morbidity and mortality worldwide. Despite pharmacologic interventions and efforts to create awareness on the importance of healthy lifestyles, CVD and associated risk factors – hypertension, diabetes, and obesity – remain among the most resilient health problems.

In addition to lifestyle factors -smoking, sedentarism, unhealthy diets- and the genetic background of the individual, decades of research have evidenced the key role of fetal and perinatal

life on the development of CVD. This phenomenon is known as *fetal programming of disease* or *Developmental Origins of Health and Disease (DOHaD)*. The fetal programming hypothesis was proposed by Dr. Barker based on epidemiologic studies demonstrating the association between low birth weight (LBW), as a consequence of poor maternal nutrition, and development of coronary heart disease and hypertension in adult life (Forsdahl, 1977; Barker and Osmond, 1986; Barker and Osmond, 1988). The hypothesis has been widely validated by means of numerous epidemiologic studies in populations exposed to starvation in different parts of the world, which confirm the association between an adverse fetal environment and increased risk of hypertension, diabetes and obesity in adult life (Stein et al., 1996; Roseboom et al., 2000; Hult et al., 2010; Wu et al., 2017). The responses initiated *in utero* by undernutrition are also modulated during the perinatal period. Thus, it has been observed that an accelerated perinatal growth trajectory in individuals born with LBW has an additional negative impact, acting as a second hit, further contributing to CVD programming (Eriksson et al., 2000; Law et al., 2002; Singhal and Lucas, 2004; Leunissen et al., 2012).

Fetal Stress Factors Implicated in DOHaD

Together with undernutrition, other stress factors that perturb the intrauterine environment have also been associated with inadequate fetal growth and subsequent development of CVD. One of these stress factors is fetal overnutrition – another form of malnutrition- due to Gestational Diabetes Mellitus (GDM), maternal obesity or excess weight gain during gestation. Obstetric complications also compromise intrauterine environment, particularly those related to placental insufficiency, like pre-eclampsia (PE), which reduce blood flow and nutrient and oxygen access to the fetus. A third factor interfering with fetal growth is exposure to environmental pollutants or toxic substances related to lifestyle, such as tobacco and alcohol. Fetal development is also affected by excess glucocorticoids, either due to maternal pharmacologic treatments or alterations in 11-β-Hydroxysteroid dehydrogenase (11-β-HSD), the placental enzymatic barrier limiting access of cortisol to the fetus.

Epidemiologic and experimental animal studies have demonstrated that all the above mentioned stress factors alter placental function. Thus, the placenta appears to play a central role in fetal programming, as the interface between the adverse intrauterine environment and the fetus (Alexander et al., 2015; Morton et al., 2016). A summary of the stress factors implicated in fetal programming is depicted in **Figure 1**.

Many of these stress factors are prevalent nowadays and fetal programming will likely contribute to the burden of cardiovascular and metabolic diseases in the next generations. Firstly, undernutrition is a chronic problem still prevalent in many parts of the world. Prematurity is another worldwide rising problem (Blencowe et al., 2013) which has been linked to CVD risk in early life. A recent study in a large cohort including over 2 million individuals born in Sweden from 1987 to 2012, found a strong association between preterm birth and incident heart failure in childhood and young adulthood (Carr et al., 2017).

Obstetric-related pathologies are also a global rising problem, particularly PE; in low-income countries associated with deficient diets and adolescent pregnancies and in high-income societies mainly due to the increase in maternity age (Ramiro-Cortijo et al., 2017). PE is associated with fetal growth restriction (FGR). Epidemiological evidence indicates that children born from pre-eclamptic mothers exhibit higher blood pressure levels (Paauw et al., 2017). FGR is also strongly associated with fetal cardiac and arterial remodeling and a subclinical state of cardiovascular dysfunction (Crispi et al., 2018) and there is also evidence of increased risk of stroke (Kajantie et al., 2009).

Maternal obesity, GDM and unbalanced diets are also widespread in high-income countries, leading to macrosomic neonates, also at risk of cardiometabolic programming. In a cohort longitudinal study, Boney et al. (2005) demonstrated that large for gestational age offspring of mothers with GDM or obesity were at significant risk of developing metabolic syndrome in childhood. It has also been shown that maternal obesity itself is another risk factor for early overweight (Gillman et al., 2003). These data suggest that the nutritional and metabolic environment of the mother may permanently program the offspring toward the development of metabolic syndrome (Xita and Tsatsoulis, 2010).

Mechanisms Implicated in Fetal Programming of CVD

Experimental animal studies have provided support to the DOHaD hypothesis and demonstrated the mechanisms implicated. They have also enabled exploring possible targets for therapeutic interventions and to conduct longitudinal studies to evaluate the effect of aging. It has been found that different fetal stress factors exhibit some common mechanisms leading to hypertension and heart disease, with slight differences in their relative implication depending on the stress factor. The following have been consistently demonstrated: (1) deficient kidney development leading to reduced nephron number and inadequate Na^+ handling, (2) alterations in blood vessels, including remodeling, stiffening and endothelial dysfunction and (3) heart alterations, namely lower cardiomyocyte number followed by fibrosis and hypertrophy, leading to cardiac dysfunction. Animal studies have also consistently demonstrated the implication of the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system, and the hypothalamic-pituitary-adrenal axis (HPA axis) in fetal programming of CVD. In addition, there is growing evidence that some of the above-mentioned alterations are mediated by epigenetic modulation of the expression of genes implicated in growth and cardiovascular control. A summary of the main mechanisms associated with fetal programming of CVD are shown in **Figure 2**. Animal studies have also evidenced a sexual dimorphism in fetal programming of CVD and demonstrated that females exhibit some protection, particularly against hypertension development. However, it is not established whether women are at lower risk than men and additional studies are needed to fully address the impact of sex on DOHaD (Alexander et al., 2014).

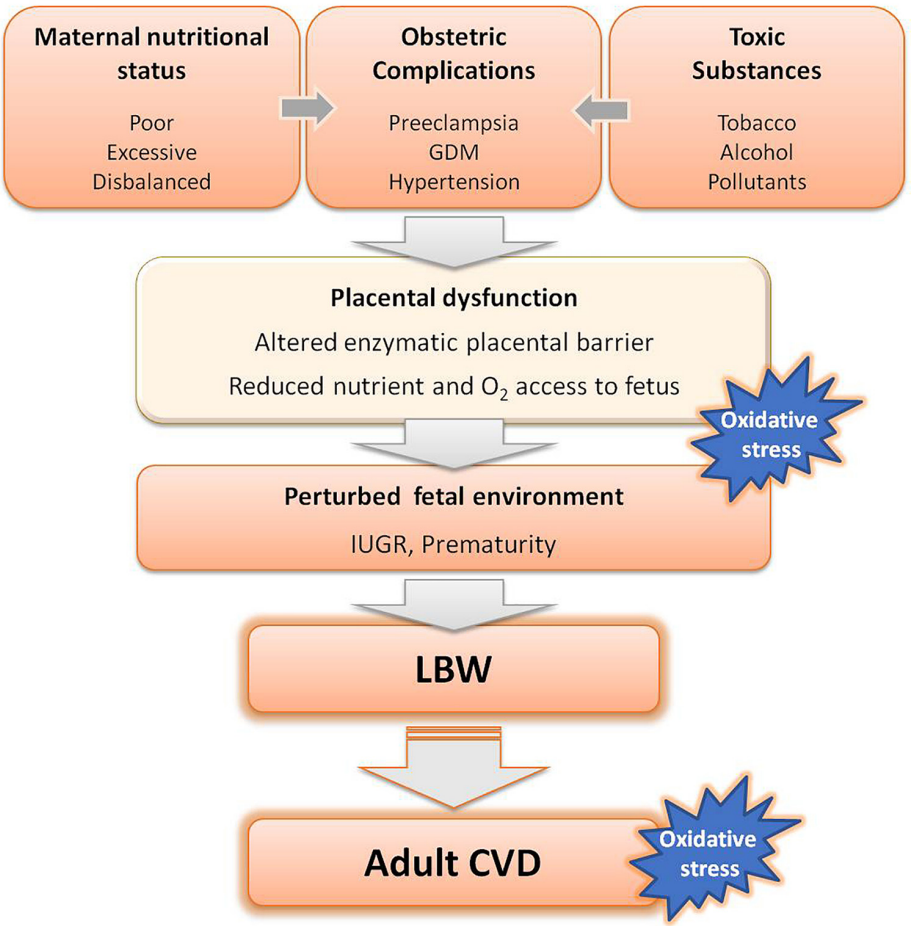

FIGURE 1 | Adverse factors known to interfere with fetal growth leading to low birth weight (LBW) and subsequent development of cardiovascular diseases (CVD) in adult life. GDM, gestational diabetes mellitus.

OXIDATIVE STRESS AND DOHaD

Oxidative stress is the basis of many adult diseases, including CVD. Oxidative stress can be defined as an imbalance between the production and elimination of reactive species, in favor of the first, leading to oxidative damage to macromolecules. Relevant reactive species participating in oxidative stress in biological systems are reactive oxygen species (ROS) and nitrogen oxygen species (RNS). These substances are inevitably produced because of aerobic metabolism and are highly reactive, particularly free radicals. Therefore, biological systems have developed a network of antioxidants to maintain redox balance. The variety of antioxidants is very large, including hormones -such as melatonin- enzymatic systems and vitamins and other low molecular weight substances which can be endogenously produced or obtained from the diet. Their coordinated actions and specific location within cells and in biological fluids, enables keeping ROS and RNS at optimum concentrations to maintain a redox balance. However, an overproduction of ROS or a deficiency of antioxidants, lead to a pro-oxidative cellular state, which damages macromolecules. A summary of the redox balance is depicted in **Figure 3**. It is worth mentioning that ROS cannot be always considered "harmful substances," neither can antioxidants be taken as "protective molecules." ROS play numerous physiological roles in cell defense, metabolism, growth and differentiation (Valko et al., 2007). An excess of antioxidants may not always confer additional protection, since they can transform into pro-oxidant molecules, depending on the nature and stability of the antioxidant and the biochemical environment (Halliwell, 2013).

During pregnancy, ROS exert several important roles; they participate in placentation and serve as signaling molecules, inducing the transcription of several genes. Therefore, they are required to allow for the normal progression of embryonic and fetal development. It must be considered that the embryo and fetus have low antioxidant capacity. Therefore, an excess production in ROS during the intrauterine period would lead to a pro-oxidative state compromising fetal growth (Dennery, 2010). Indeed, numerous data suggest that oxidative stress is at the basis of obstetric and fetal complications associated with LBW. Furthermore, oxidative stress has also been proposed as the underlying mechanism between inadequate fetal development

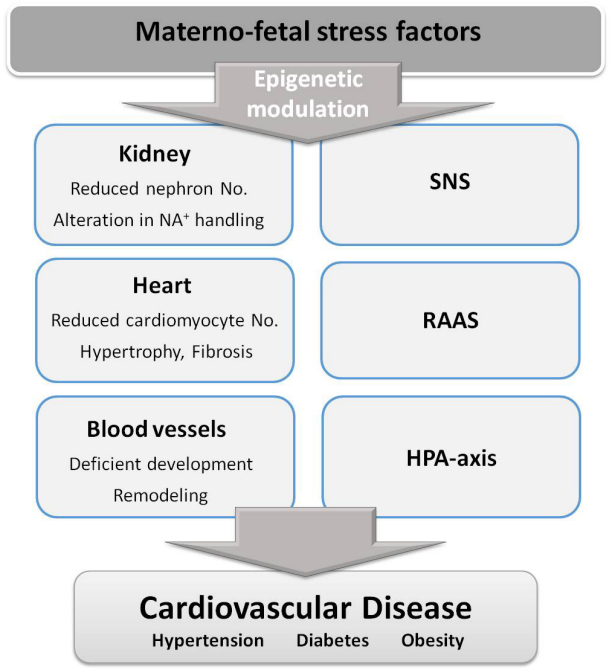

FIGURE 2 | Stress factors during fetal life alter several organs and physiological systems, through epigenetic changes, contributing to cardiovascular disease development. SNS, sympathetic nervous system; RAAS, renin-angiotensin-aldosterone system; HPA-axis, hypothalamic-pituitary-adrenal axis.

and programming of CVD at later stages of life. This review aims to cover these two aspects emphasizing the role of the placenta and redox alterations in key organs responsible for cardiovascular control.

Association Between Suboptimal Fetal Conditions, Oxidative Stress and LBW

Oxidative Stress and Undernutrition

Pregnancy requires an increased intake of macro and micronutrients for maternal and fetal needs and maternal malnourishment is well proven to lead to LBW and adverse perinatal outcomes. In fact, most LBW infants are born to undernourished mothers (World Health Organization [WHO], 2014). Even if maternal diet has sufficient macronutrients, micronutrient deficiencies, particularly iron, are very common in many countries (Black et al., 2013). Many micronutrients are co-factors of antioxidant enzymatic systems or are antioxidants themselves. Therefore, in mothers suffering malnutrition antioxidant deficiency may generate a situation of oxidative stress. Intervention strategies in undernourished pregnant women demonstrate that micronutrient supplementation reduces the risk of LBW (Ota et al., 2015) and improves the metabolic health of children (Ekström et al., 2016). Undernutrition has an impact on the placenta, leading to deficient development or reducing the enzymatic barrier 11-β-HSD, allowing for excess cortisol reaching the fetus. The catabolic actions of cortisol are associated with increased ROS production. Placental alterations induced by undernutrition are also associated with oxidative stress. Thus, exposure to low protein diet during pregnancy induces an increase in oxidative stress biomarkers in the placenta and metabolic dysfunction in the offspring; alterations which were prevented by treatment with the antioxidant Resveratrol (Vega et al., 2016). In pregnant women with deficient diets, antioxidant supplementation also improved the metabolic health of children, but no differences in oxidative stress biomarkers were found, either in the mother or in the offspring (Ekström et al., 2016). Our revision of the literature indicates that the relationship between maternal undernutrition, maternal oxidative stress and offspring cardiometabolic outcome remains insufficiently explored in humans.

Oxidative Stress and Gestational Complications

Although the pathophysiology of PE remains incompletely understood, there is a general agreement that a deficient placental vasculature and oxidative stress play important roles. A poor development of placental vessels disrupts the normal pattern of blood flow, reducing nutrient access to the fetus and is a potential risk of ischemia/reperfusion, excess ROS production and injury (Burton, 2009; Burton et al., 2009). In fact, increased biomarkers of oxidative damage to lipids and proteins has been found in plasma (Bernardi et al., 2008) and in the placenta from pre-eclamptic women and in HELLP syndrome, also related to PE (Zusterzeel et al., 2001).

Pregnancy-related oxidative stress might also result from a poor maternal antioxidant status, insufficient to counteract the physiological ROS elevation. This is particularly important around weeks 10–12 of gestation, when maternal circulation is established and oxygen rises within the placenta. In fact, oxidative damage in pre-eclamptic women has been suggested to be the consequence of insufficient endogenous antioxidants (Zusterzeel et al., 2001; Llurba et al., 2004). We have evidence that poor maternal plasma antioxidant status in the first trimester of gestation is associated with the subsequent development of an obstetric complication (Ramiro-Cortijo et al., 2016). An important antioxidant molecule during pregnancy is melatonin, a hormone with pleiotropic actions (Chen et al., 2013), both as a direct free radical scavenger (Reiter et al., 2000), and stimulating the expression of enzymatic antioxidants (Rodriguez et al., 2004). The role of melatonin on the feto-placental unit is supported by the observed decrease in melatonin levels and in receptor expression in the placenta from women with PE (Gitto et al., 2004; Lanoix et al., 2012). In a cohort of healthy women at week 10 of pregnancy, we have found a negative correlation between plasma levels of melatonin and carbonyls, supporting the protective role of this hormone against protein oxidation (Ramiro-Cortijo et al., 2016). Recent animal studies in models of developmental programming have evidenced the potential implication of melatonin as a reprogramming strategy to prevent DOHaD-related diseases (Tain et al., 2017). Future human studies and clinical trials are needed to translate this information from animal models.

Maternal oxidative stress not only has an impact on maternal health, but may compromise fetal development, either directly

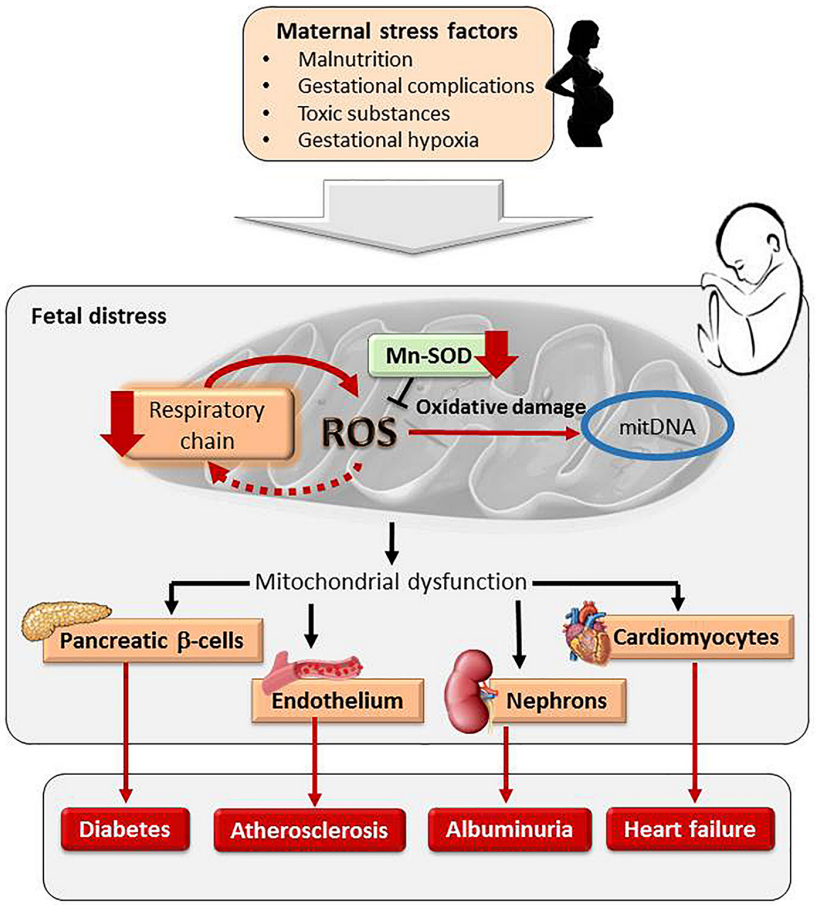

FIGURE 3 | Maternal exposure to stress factors during pregnancy programs fetal mitochondria. An increase in ROS, respiratory chain inhibition and a vicious circle of ROS-induced ROS release and a reduction of mitochondrial antioxidant system MnSOD damages mitDNA. Mitochondrial programming in response to intrauterine stress factors has been found in pancreatic β-cells, cardiomyocytes, vascular endothelium and nephrons, contributing to cardiometabolic diseases.

or indirectly through reduction in placental perfusion and fetal nutrition (Karowicz-Bilinska et al., 2007; Hracsko et al., 2008). Increased biomarkers of oxidation have been found elevated in maternal and cord plasma from pregnant women carrying FGR fetuses (Biri et al., 2007; Kimura et al., 2013). Oxidative damage to the placenta has been proposed to be responsible for fetal growth restriction, as demonstrated by the higher placental levels of 8-OHdG -a marker of DNA oxidative damage- in pre-eclamptic pregnancies with FGR compared to those without fetal growth compromise (Fujimaki et al., 2011). Hypoxia may be implicated in these alterations as shown by the elevation in HIF-1 in the placenta from pre-eclamptic women together with higher levels of DNA oxidative damage and fetal growth restriction (Kimura et al., 2013).

Oxidative stress in LBW infants is also associated with CVD risk factors in early life. A close relationship between lipid peroxidation levels and cardiometabolic alterations -high blood pressure, altered lipid profiles and insulin resistance- has been found in children born FGR or small for gestational age (Franco et al., 2007; Mohn et al., 2007; Chiavaroli et al., 2009).

Premature infants are another group at risk of programming of CVD where oxidative stress has been shown to play a role. They are born with LBW before organogenesis is complete and they are commonly exposed to several stress factors known to be implicated in DOHaD, namely inadequate nutritional status or exposure to glucocorticoids. Epidemiologic studies reveal that preterm neonates exhibit hypertension in adolescence and as young adults (de Jong et al., 2012). Hypertension development in individuals born premature is associated to alterations in RAAS and sympathetic nervous system, as well as to renal and vascular abnormalities (Sutherland et al., 2014), i.e., through similar mechanisms to those found in fetal programming. Oxidative stress is also a strong contender in the association between prematurity and high blood pressure development. Preterm infants are especially vulnerable to ROS due to immaturity and insufficient antioxidants (Shim and Kim, 2013). Low levels of vitamins E and A and catalase have been reported in preterm infants, associated with increased levels of lipid and protein oxidation (Negi et al., 2012; Abdel Ghany et al., 2016). Moreover, preterm infants are exposed to catheters, mechanical ventilation, which may induce high levels of ROS.

Thus, the imbalance between ROS production and antioxidant defenses may contribute not only to some common diseases of prematurity (Shim and Kim, 2013), but also to long tern development of cardiovascular and metabolic diseases.

Oxidative Stress in Response to Toxic Substances

The fetus is a potential target for toxics, including substances related to lifestyle factors, such as tobacco and alcohol, as well as environmental pollutants. These substances cross the placenta and are known to compromise development, particularly in the embryo. In addition, there is increasing evidence that they may also program the fetus for later development of CVD.

The negative effects of tobacco consumption during pregnancy on fetal development are well documented. Smoking is associated with preterm birth and FGR (Leonardi-Bee et al., 2008; Banderali et al., 2015). Even low cigarette consumption during pregnancy seems to have an effect on birth weight (Berlin et al., 2017). Furthermore, there is also evidence that smoking programs the offspring to hypertension, already observed in childhood, through endothelial dysfunction and renal alterations (Banderali et al., 2015). The mechanisms implicated in this association have been explored in experimental animals and suggest that tobacco may induce oxidative stress in the developing organs (Al-Gubory, 2016). Several components of tobacco may contribute. The first is CO, which can induce a hypoxic effect due to combination with hemoglobin (Banderali et al., 2015). CO has also been associated with alterations in the vasculature, leading to a reduction in blood flow and fetal nutrient compromise. For example, higher indices of CO in exhaled air are associated with increased resistance of umbilical arteries (Machado et al., 2011). Furthermore, smoking in pregnancy is linked to umbilical cord structural alterations and advance oxidation products (Rua Ede et al., 2014). Finally, CO might also compromise normal mitochondrial function due to its affinity to mitochondrial respiratory chain complex IV (Garrabou et al., 2016). Another substance in tobacco associated with fetal programming is nicotine, which exerts several negative effects in pregnancy, such as reducing uterine arterial blood flow (Al-Gubory, 2016) and promotes vascular oxidative stress and dysfunction in the offspring (Bruin et al., 2010). Nicotine replacement therapy has been developed as a pharmacotherapy for smoking cessation, but safety during pregnancy and the long-term effects on the progeny remains to be determined (Lim and Sobey, 2011).

Alcohol consumption during pregnancy has detrimental effects on placental formation and fetal growth (Kalisch-Smith and Moritz, 2017). In western countries, alcohol consumption is widespread in women of reproductive age, although they often stop drinking after discovering pregnancy. The first trimester appears to be the most sensitive period and there is also evidence that alcohol might have harmful effects even during the peri-conceptional period, increasing the risk of LBW and prematurity (Nykjaer et al., 2014). Alcohol effects are also linked to oxidative processes; ROS increases, depletion of antioxidants and elevation of biomarkers of oxidative damage have been reported in association with FGR (Nogales et al., 2017). Moreover, alcohol may lead to malnutrition and to selenium and folate deficiency (Ojeda et al., 2017), which are also linked to inadequate fetal development. These data point out the importance of population health policies, including educational programs to minimize tobacco and alcohol exposure, not only during pregnancy, but also during the peri-conceptional period.

While alcohol and tobacco can be voluntarily avoided, environmental pollutants are sometimes difficult or even impossible to evade. In large concentrations, these chemicals are teratogens. However, exposure to small doses during gestational periods might also interfere with fetal growth. Even if a single component might not induce an adverse effect, exposure to multiple contaminants has cumulative adverse outcomes, even at low concentrations (Al-Gubory, 2016).

Oxidative stress is also at the basis of the detrimental effects of fetal exposure to environmental toxics and their association with LBW and CVD programming. There is evidence of an inverse relationship between birth weight and concentration of pesticides in the placenta, maternal blood, cord blood, and breast milk (Lopez-Espinosa et al., 2007; Dewan et al., 2013). LBW also appears to be induced by exposure to polychlorinated biphenyls and heavy metals. Most environmental toxics induce abnormal ROS generation and oxidative stress, being a key target mitochondrial DNA oxidative damage (Al-Gubory, 2014, 2016). Other toxic substance is the halogen bromine, which is extensively used in industry. Experimental studies in mice have demonstrated that exposure to this oxidant gas during gestation induces blood pressure elevation in the dam, together with abnormal placental development. It was also found severe FGR, systemic inflammation and evidence of pulmonary and cardiac injury in the offspring. Since these are features similar to those found in PE, it has been suggested that it would be important to monitor pregnant women exposed to this toxic substance (Lambert et al., 2017).

Exposure to noxious substances during pregnancy are not only deleterious for cardiovascular function, but also program the fetus for respiratory diseases (Maritz et al., 2005; Petre et al., 2011; Harding and Maritz, 2012). In this sense, there is increasing evidence that chronic obstructive pulmonary disease (Stocks and Sonnappa, 2013) and asthma (De Luca et al., 2010) are associated with insults to the developing lung during fetal and early postnatal life when lung growth and development are rapid. Altered gene expression, through epigenetic modifications by environmental stress factors are among the most plausible mechanisms (Henderson and Warner, 2012; Jilling et al., 2017).

Oxidative Stress and Mechanisms Implicated in Fetal Programming of CVD

Oxidative Stress-Induced Organ Damage

Oxidative damage in the kidney, blood vessels, and the heart are well known to contribute to organ dysfunction leading to CVD. Similar alterations have been found in animals exposed to different suboptimal intrauterine conditions, providing support for oxidative stress as a common underlying mechanism.

Kidney

Exposure to undernutrition, excess glucocorticoids, placental insufficiency or toxic substances during fetal life lead to

alterations in nephrogenesis, Renin-Angiotensin-System (RAS), Na^+ excretion and other renal alterations which contribute to hypertension (Paixão and Alexander, 2013). Alterations during the perinatal period are also important, particularly in species, such as rodents, where nephrogenesis continues after birth. Some of the kidney alterations are related to oxidative damage, as supported by the increase in renal markers of oxidative stress found in various animal models of developmental insult (Vehaskari et al., 2001; Stewart et al., 2005; Cambonie et al., 2007; Ojeda et al., 2007; Vieira-Filho et al., 2013). Some of the oxidative stress-related kidney alterations are linked to the RAAS dysfunction. Increased renal 8-isoprostane in response to Ang II (Bi et al., 2014), alterations in Ang II AT1/AT2 receptor subtypes and increased ROS-mediated Ang II responses are observed in association with hypertension development in animals exposed to fetal stress factors (Gwathmey et al., 2011).

Blood vessels

Oxidative stress is also a strong candidate contributing to the vascular alterations, particularly endothelial dysfunction, observed in animal models of fetal programming (Alexander et al., 2015; Morton et al., 2016). Both increased ROS/RNS production or defective antioxidants have been reported. There is evidence that increased superoxide generation, via activation of NADPH pathways, may contribute to vascular hyper-reactivity (Zhu et al., 2016). Vascular nitric oxide (NO) destruction by superoxide anion is one of the alterations consistently found in animals exposed to various fetal stress factors, namely nutrient deficiency, hypoxia, excess glucocorticoids, or placental insufficiency (Thompson and Al-Hasan, 2012). Excess superoxide anion interacts with NO, reducing its bioavailability and generating ONOO, a powerful oxidant which uncouples eNOS through oxidation of BH4, creating a vicious circle of superoxide anion generation (Morton et al., 2016).

Oxidative damage might also result from decreased antioxidants. We have evidence of a plasma deficiency in thiols, GSH, SOD, and melatonin, in pre-puberal rats exposed to fetal nutrient restriction (Rodríguez-Rodríguez et al., 2015). Plasma and tissue antioxidant deficiencies have also been found in goats exposed *in utero* to protein restriction (He et al., 2012) and SOD activity is reduced in the aorta of animals exposed to nicotine during fetal life (Xiao et al., 2011). The presence of oxidative damage prior to blood pressure elevation, supports a role of redox disbalance as a causative element implicated in fetal programming of hypertension (Rodríguez-Rodríguez et al., 2015).

Heart

Experimental animals of DOHaD provide evidence that hypoxia, nutrient deficiency or other stressors during intrauterine life, induce left ventricular hypertrophy (Gezmish et al., 2010; Gray et al., 2014; Zohdi et al., 2014; Rodríguez-Rodríguez et al., 2017). Animals exposed to fetal stress factors also exhibit a larger susceptibility to develop arrhythmias (Hu et al., 2000) and ischemia-reperfusion injury (Xu et al., 2006; Elmes et al., 2007).

The heart is the organ with the highest oxygen uptake and largest density of mitochondria and it is an active source of ROS. Therefore, the heart is a potential target of oxidative damage in situations of redox disbalance, such as those induced by stress factors during intrauterine life. Several lines of research support this hypothesis. Sheep exposed to dexamethasone *in utero* exhibit increased coronary artery ROS production (Roghair et al., 2008). Prenatal hypoxia also generates oxidative stress in fetal hearts in a variety of animal species (Thompson and Al-Hasan, 2012). Cardiac hypertrophy in response to nutrient restriction during fetal life is associated with increased heart NADPH oxidase expression prior to hypertension development (Rodríguez-Rodríguez et al., 2017) and oxidative and nitrosative damage (Rueda-Clausen et al., 2012; Tarry-Adkins et al., 2013).

Numerous experimental studies evidence a sexual-dimorphic response and a certain degree of protection in females, which do not develop hypertension or develop milder forms, (Alexander et al., 2014). Oxidative stress seems to contribute to the sex differences in fetal programming. For example, there is evidence of sex specific programming of the RAAS and subsequent ROS increase in association with hypertension development (Alexander et al., 2014). Antioxidant deficiency in animal models of fetal programming also seem to be sex-dependent. Thus, expression and activity of renal antioxidants are increased in female, but not in male offspring exposed to several fetal stress factors, namely placental insufficiency (Ojeda et al., 2013), betamethasone exposure (Bi et al., 2014) or undernutrition (Rodríguez-Rodríguez et al., 2015). A possible mechanism explaining the sexual dimorphism in fetal programming is the protective effects of estrogens, evidenced by the effects of castration or ovariectomy in experimental animal models (Alexander et al., 2014). Alternative mechanisms include a more adaptive growth strategies under stress conditions of females (Eriksson et al., 2000) or a better adaptation of the female placenta to suboptimal conditions (Rosenfeld, 2015).

Antioxidant Treatments

The implication of oxidative stress in fetal programming is also evidenced by the prevention of hypertension and organ damage by prenatal antioxidant treatment. Thus, increased Ang II-mediated contractile response in offspring from rats exposed to protein restriction during gestation is abolished by treatment of the dams with Lazaroid, a lipid peroxidation inhibitor, which also prevented the rise in blood pressure (Cambonie et al., 2007). Vitamin C and E treatments reversed the increased vasoconstrictor responses observed in animals exposed to postnatal glucocorticoids (Herrera et al., 2010) or to fetal hypoxia (Thakor et al., 2010). Similar effects are also observed with Allopurinol, a xanthine oxidase inhibitor (Kane et al., 2014). Antioxidant treatments also prevent renal or cardiovascular damage in response to placental insufficiency (Silva et al., 2011), prenatal glucocorticoid exposure (Roghair et al., 2011) or various models of undernutrition during fetal life (Elmes et al., 2007; Rexhaj et al., 2011; Vieira-Filho et al., 2011).

The above mentioned findings in animal models have encouraged clinical trials exploring the possible benefits of antioxidant therapy in compromised pregnancies. Unfortunately, results have not been as promising as expected. Some have failed to demonstrate improved pregnancy or fetal outcomes and,

worryingly, several have demonstrated increased complications (Morton et al., 2016). This antioxidant paradox has been accounted for by Halliwell (2013) by means of various facts. On the one hand, laboratory animals seem more responsive to dietary antioxidants than humans. Secondly, some antioxidants (e.g., polyphenols and ascorbate) could exert mild pro-oxidant effects under some circumstances, related to the presence of transition metals. Thirdly, many clinical trials with antioxidants have not taken into consideration the baseline nutritional status of the population (Halliwell, 2013). In fact, this could account for the beneficial effects of supplements in undernourished populations, but not in those with normal nutritional status. It has also been proposed that mild pro-oxidant effects could even be beneficial, perhaps by increasing the levels of antioxidant defenses (Halliwell, 2013). In this sense moderate physical training could represent an alternative to pharmacological treatments. It is well known that moderate exercise appears to be beneficial for health, in part due to ROS-induced antioxidant defense systems (Pingitore et al., 2015). These aspects clearly warrant further investigation.

Mitochondria Programming

The role of the mitochondria in fetal programming is gaining attention. In hostile fetal environments -such as in situations of limited access to nutrients and oxygen- "mitochondria programming" might be a strategy for cell survival, but with later detrimental effects. For example, fetal hypoxia (induced by placental insufficiency or in high altitude pregnancies) has been shown to down-regulate oxidative metabolism and switch it to glycolytic pathways, along with mitochondrial dysfunction. These alterations have been suggested to participate in poor fetal growth leading to LBW, which is frequently found in pre-eclampsia and gestations at high altitude (Murray, 2012).

Mitochondria are the powerhouse that provides energy for cell function; therefore, a reduction in number or a functional alteration are likely to be detrimental for cells, particularly for those with high energy requirements such as pancreatic β-cells or cardiomyocytes. Alteration in β-cell mitochondrial function has been observed in animal models of undernutrition or placental insufficiency and has been proposed to explain the development of diabetes in later life (Simmons et al., 2005; Reusens et al., 2011). Similarly, a decrease in mitochondrial energetics has been found in skeletal and cardiac muscles of adult offspring from undernourished mothers and suggested to represent a compensatory mechanism programmed *in utero* to handle limited nutrient availability (Beauchamp and Harper, 2016). Decreased mitochondrial respiration rates are also a characteristic of heart failure. Therefore, it is possible that mitochondrial programming might provide the basis for the cardiac dysfunction observed in animal models of DOHaD (Rueda-Clausen et al., 2008; Rodríguez-Rodríguez et al., 2017).

Oxidative stress may contribute to mitochondrial dysfunction. During cellular respiration ROS are inevitably produced, but redox balance is maintained by elimination via Mn-SOD. If electrons leaking into the mitochondrial space are in excess the antioxidant system cannot eliminate the ROS overload. This situation might occur if oxidative phosphorylation is inhibited or if MnSOD is reduced. An excess ROS has deleterious consequences for the mitochondria. ROS inhibit electron transport chain activity leading to a vicious circle of ROS-induced ROS release (Zorov et al., 2006). On the other hand, ROS damage mitochondrial DNA (mitDNA), which has a limited repair system and lacks protective histone proteins (Reusens et al., 2011). Evidence of alterations in mitDNA has been reported in human and animal models of fetal programming. In this sense, umbilical cords from small for gestational age babies have lower mitDNA content (Sookoian et al., 2013). Alterations in mitDNA from β-cells have also been found in animal models of placental insufficiency and maternal undernutrition, in association with later development of diabetes (Simmons et al., 2005; Reusens et al., 2011). Mitochondria dysfunction in endothelial cells has also been proposed to be implicated in fetal programming of atherosclerosis (Leduc et al., 2010). There is also substantial evidence from human studies and animal models that environmental contaminants, alcohol and tobacco also induce mitDNA damage via ROS (Al-Gubory, 2014). For example, tobacco exposure during gestation in mice leads increased renal levels of mitochondrial-derived ROS in the progeny, a reduction in oxidative phosphorylation proteins and in the activity of mitochondrial MnSOD. These alterations preceded the onset of albuminuria (Stangenberg et al., 2015). A summary of the relationship between maternal stress factors, mitochondrial programming and cardiometabolic disease is shown in **Figure 3**.

The above-mentioned evidence supports the hypothesis that mitochondrial damage ("programming") induced by suboptimal conditions during intrauterine life might provide the background for subsequent development of cardiometabolic diseases. However, there are many aspects still unresolved. Firstly, mitochondria are not homogeneous in cells and their response might differ depending on the type of stress factor, the tissue implicated and the time of insult. In addition, mitochondria are highly dynamic organelles and their activity can be regulated by fusion and migration (Chan, 2006). It has been found that mitochondrial dynamics can influence the response of the organelle to oxidative stress (Park and Choi, 2012). Furthermore, the response of mitochondria to fetal stressors appears to be modulated by sex, an important aspect in fetal programming which deserves further attention.

Epigenetic Modulation and Cell Senescence

Epigenetic modifications, i.e., heritable changes in gene expression that do not result from alterations in the nucleotide sequence, regulate cellular differentiation and fetal development. A mounting body of evidence implicates epigenetic modulation -DNA methylation, histone acetylation, or microRNA- as fundamental mechanism implicated in DOHaD and in the transmission of alterations to future generations.

The genes involved are starting to be revealed. Among others, RAS and NO pathways have been found altered in humans born LBW and in animal models of fetal programming.

Hypomethylation of AT1b receptor has been evidenced in rodents exposed to fetal undernutrition (Bogdarina et al., 2007). Similarly, hypomethylation of the Angiotensin Converting Enzyme promoter was found in children born small for gestational age and related to blood pressure (Rangel et al., 2014). On the other hand, hypomethylation of eNOS promoter has been found in human umbilical endothelium from FGR fetuses (Krause et al., 2013).

Alterations in histone deacetylation processes are also implicated in fetal programming (Anwar et al., 2016). In this regard, sirtuins (SIRTs) may play a key role. SIRTs are a family of deacetylases and key regulators of vascular homeostasis. The most extensively studied is SIRT1, which regulate, among other processes, inflammation, endothelial function, mitochondrial biogenesis and aging. Oxidative stress might be a key player, since it reduces the histone deacetylation activity of SIRT1 (Hwang et al., 2013).

SIRT1 also preserves the normal telomere length and therefore, changes in its activity can be implicated in cellular senescence. Several common risk factors for CVD such as smoking, diabetes mellitus, hypertension, obesity and alcohol consumption, have been associated with short telomere length (Yeh and Wang, 2016). However, causality remains undetermined (Fyhrquist et al., 2013). Tissue inflammation and oxidative stress have been proposed as potential mechanisms implicated (Yeh and Wang, 2016; Vasconcelos-Moreno et al., 2017). Cellular senescence may also be programmed, as evidenced by several studies which have found an association between adverse intrauterine environment and shorter telomere length later in life (Entringer et al., 2011; Hallows et al., 2012; Tarry-Adkins and Ozanne, 2014), also observed in neonates with abnormal fetal growth (Tellechea et al., 2015). Among possible mechanisms inflammation, oxidative stress and energy failure due to mitochondrial dysfunction have been proposed (Von Zglinicki, 2002; de Almeida et al., 2017).

Epigenetic dysregulation induced by stress factors during intrauterine life, not only lead to CVD in the adult, but are also transferable to the next generation (Patel and Srinivasan, 2002). Fortunately, epigenetic modifications are reversible and interventions which modify epigenetic programming may represent an important and novel approach to reduce the burden of CVD (Anwar et al., 2016).

SUMMARY AND CONCLUSIONS

Environmental stress factors during fetal and perinatal life can shape the future health of the individual and increase susceptibility to several adult diseases. Suboptimal intrauterine conditions induce alterations in placental redox balance, associated with poor fetal development. Furthermore, oxidative stress is also at the basis of changes in the kidney, heart, blood vessels, and cardiovascular control systems, ultimately leading to disease. There are some disparities, such as time of initiation of redox alteration, the implication of different antioxidants and reactive species or the organs which are more susceptible to oxidative damage. These differences may depend on the type of fetal stress factor, vulnerability of the gestational period, or age of study of the cardiovascular damage. In addition, sex appears to play an important role in the susceptibility to fetal programming, but the mechanisms behind it and the implication of oxidative stress are still not sufficiently explored. Therefore, additional research is needed to gain insight into how specific stress factors throughout pregnancy and perinatal life modulate organ growth, to develop specific strategies to reduce their impact on disease.

Another important aspect is that obstetric and fetal complications, malnutrition and exposure to toxic substances - well known fetal stress factors- are growing problems worldwide. Therefore, it is foreseen that fetal programming will contribute to the burden of CVDs in future generations. Epigenetic modulation of key genes implicated in cardiometabolic control seem to be at the origin of fetal programming. Therefore, interventions which modulate gene expression may represent a possible approach to counteract the deleterious effects of adverse intrauterine environment. In addition to the development of therapeutic interventions, other possible strategies are implementation of governmental policies aimed at reducing malnutrition or environmental pollutants and at promoting healthy lifestyles during gestation by means of education.

AUTHOR CONTRIBUTIONS

PR-R, DR-C, and CR-H searched and organized the data. PR-R, DR-C, CR-H, ALdP, MG, and SA wrote a section of first version of the manuscript. SA, MG, and ALdP wrote the final version. All authors contributed to manuscript revision, read and approved the submitted version.

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