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Review article

Dual roles of vascular-derived reactive oxygen species—With a special reference to hydrogen peroxide and cyclophilin A—

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ABSTRACT

Reactive oxygen species (ROS) have been considered to play a major role in the pathogenesis of cardiovascular diseases. However, this notion needs to be revised since recent evidence indicates that vascular-derived hydrogen peroxide (H_2O_2) serves as an important signaling molecule in the cardiovascular system at its low physiological concentrations. At low concentrations, H_2O_2 can act as a second messenger, transducing the oxidative signal into biological responses through post-translational protein modification. These structural changes ultimately lead to altered cellular function. Intracellular redox status is closely regulated by the balance between oxidant and antioxidant systems and their imbalance can cause oxidative or reductive stress, leading to cellular damage and dysregulation. For example, excessive H_2O_2 deteriorates vascular functions and promotes vascular disease through multiple pathways. Furthermore, cyclophilin A (CyPA) has been shown to be secreted from vascular smooth muscle cells and to augment the destructive effects of ROS, linking it to the development of many cardiovascular diseases. Thus, it is important to understand the H_2O_2 signaling and the roles of downstream effectors such as CyPA in the vascular system in order to develop new therapeutic strategies for cardiovascular diseases. In this review, we will discuss the dual roles of vascular-derived H_2O_2 in mediating vascular functions (physiological roles) and promoting vascular diseases (pathological roles), with particular emphasis on the function of CyPA. This article is part of a Special Issue entitled "Redox Signalling in the Cardiovascular System".

Contents

1. Introduction

Endothelial cells and vascular smooth muscle cells (VSMC) secrete a variety of vasoactive substances that contribute to vascular protection as well as vascular remodeling [1,2]. Furthermore, the growth factors secreted from the VSMC play important roles in the remodeling process as they mediate various cellular responses [3].

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Oxidative stress is one of the important stimuli that modulate VSMC function and promote VSMC growth by inducing autocrine/paracrine growth mechanisms [4].

Oxidative stress is generated by high levels of reactive oxygen species (ROS), including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) [5]. ROS have been shown to promote cell proliferation and hypertrophy in a concentration-dependent manner [6]. Moreover, excessive ROS production can cause DNA damage and harmful protein oxidation, ultimately promoting vascular diseases. Indeed, ROS production has been implicated in the pathogenesis of cardiovascular diseases [7–9], however, its specific molecular targets remain to be elucidated.

Although high levels of ROS appear to contribute to vascular disease development, strictly controlled ROS formation has been shown to mediate many important physiological functions in the vascular cells. For example, H_2O_2 plays a crucial role as a signaling molecule at very low concentrations [10] as we have previously demonstrated that it is one of the endothelium-derived hyperpolarizing factors (EDHF) that modulate vascular tone especially in the microvessels [11–13] as well as in human coronary arteries [14]. In contrast, H_2O_2 has also been shown to induce constrictions and a biphasic effect in a concentration-dependent manner [15,16]. However, a plausible explanation for why or how ROS contribute simultaneously to both vascular protection and vascular diseases remains to be elucidated [17].

2. Regulation of ROS generation: oxidants and antioxidants

Intracellular redox status equilibrium is maintained by the balance between oxidants (e.g. ROS) and antioxidants that can scavenge ROS in the cell [18]. Excessive ROS damage mitochondrial proteins and further increases ROS levels, thus forming a vicious cycle of oxidative damage. In addition to the generation of ROS in the mitochondria, several enzymes can also generate intracellular ROS [18]. Among these enzymes, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) dynamically produce O_2^- and H_2O_2 (Fig. 1). Importantly, the production of endothelial H_2O_2 for EDHF responses appears to depend in part upon endothelial NOS (eNOS) [19]. eNOS also produces NO with a resultant production of cyclic guanosine monophosphate (cGMP) and NO can react with O_2^- to produce peroxynitrite ($ONOO^-$) (Fig. 2) [20]. Other enzymes, such as cytochrome P450 and xanthine oxidase (XO), also produce intracellular ROS under pathological conditions [18].

The amount of ROS produced by the mitochondria and oxidizing enzymes is offset by the presence of antioxidants [21]. For example, O_2^- is rapidly dismutated to H_2O_2 by superoxide dismutase (SOD) and is therefore highly restricted to certain cellular compartments (Fig. 1). Among the ROS, H_2O_2 is most stable and can penetrate the membrane and rapidly reach its cellular targets, acting as a second messenger. However, H_2O_2 is neutralized by catalase localized in the peroxisome, which catalyzes the decomposition of H_2O_2 to oxygen and water (Fig. 2). In addition, peroxiredoxins also reduce H_2O_2 levels, whereby

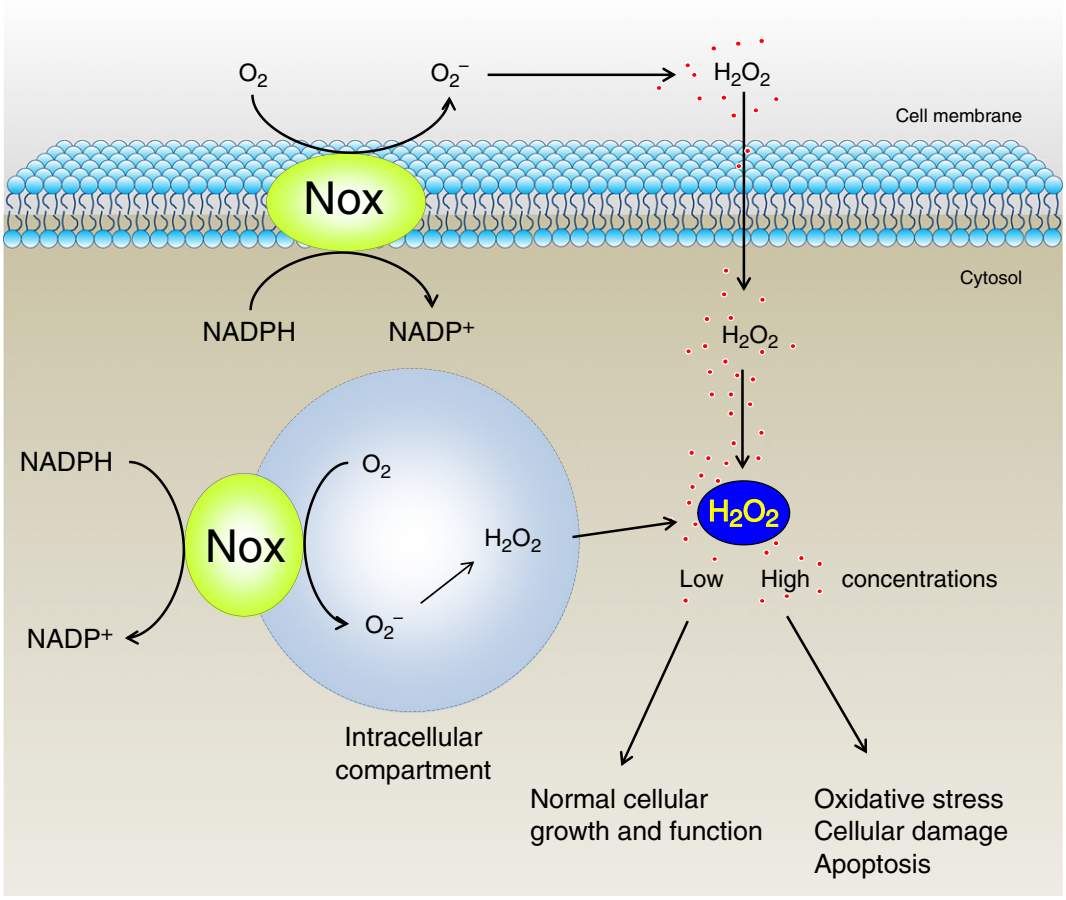

Fig. 1. H_2O_2 as a second messenger. NADPH oxidases (Nox) dynamically produce O_2^- and H_2O_2. O_2^- is very rapidly dismutated to H_2O_2 by superoxide dismutase (SOD) and is restricted to certain cellular compartments. H_2O_2 is stable and can penetrate the membrane and reach its targets rapidly, which enables it to act as a second messenger. Thus, intracellular H_2O_2 levels are lowered by passive and active diffusion into the extracellular space. The remaining H_2O_2 induces numerous biomolecular modifications, especially the oxidation of cysteine residues. At low physiological levels, H_2O_2 can promote cell growth, while at high pathological levels, it can create oxidative stress and apoptosis. NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidases.

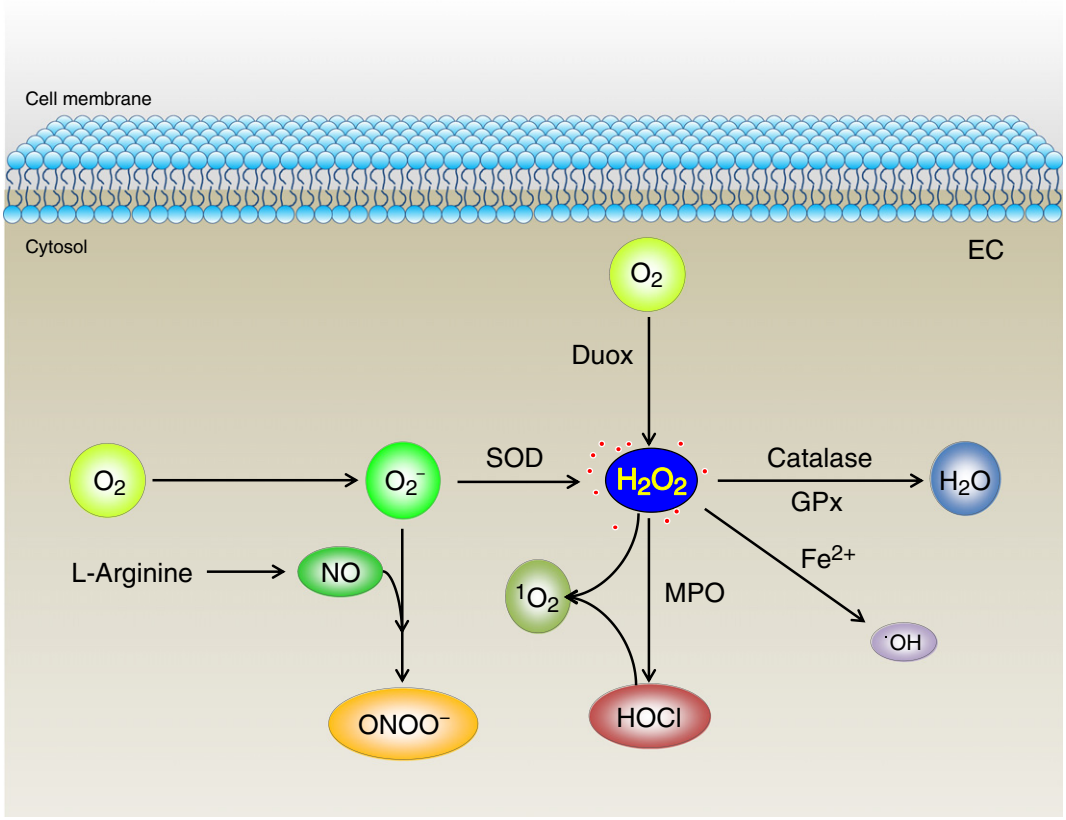

Fig. 2. Biochemical pathways of hydrogen peroxide (H_2O_2) generation and metabolism. Several enzymes can produce intracellular superoxide anions (O_2^-), such as NADPH oxidases (Nox), xanthine oxidase, aldehyde oxidase, cytochrome P450s, the electron transport chain, and urate oxidase. O_2^- is either rapidly dismutated to H_2O_2 spontaneously or catalyzed by superoxide dismutase (SOD). H_2O_2 is also formed by the catalyzed two-electron reduction of molecular oxygen (O_2) or can diffuse in from the extracellular matrix. O_2^- can also react with NO to produce peroxynitrite ($ONOO^-$). H_2O_2 is converted to other reactive oxygen species such as hydroxyl radicals ($^{\cdot}OH$, with Fe^{2+}) and hypochlorous acid (HOCl, with chloride, catalyzed by myeloperoxidase). H_2O_2 is also neutralized by catalase localized in peroxisomes, myeloperoxidase (MPO) and glutathione peroxidase (GPx). SOD, superoxide dismutase; O_2^-, superoxide anions; $ONOO^-$, peroxynitrite; $^{\cdot}OH$, hydroxyl radicals; HOCl, hypochlorous acid; MPO, myeloperoxidase; GPx, glutathione peroxidase.

the peroxidatic thiol reacts with the oxidant to form a sulfenic acid. Peroxiredoxin is then regenerated by the antioxidant protein thioredoxin 1 (Trx1), thus balancing the intracellular redox state [22]. Trx1 also works as a signaling intermediate that can sense redox state imbalances and to correct these asymmetries, transduces signals to other effectors, including transcription factors and kinases [18]. The dual roles of ROS, particularly H_2O_2 as both a protective and pathological agent, appear to be particularly important in vascular health.

3. ROS in the vascular system

3.1. Physiological levels of ROS contribute to vascular homeostasis

In the vascular wall, ROS are generated by several enzymes, including NADPH oxidases, xanthine oxidase, enzymes in the mitochondrial respiratory chain, and lipoxygenases [23]. Physiological levels of ROS regulate cell function, proliferation and normal levels of cell death. For example, at low concentrations, H_2O_2 plays an important role in endothelial function and vascular relaxation [11–13]. Endothelium-dependent relaxation is mediated primarily by prostacyclin, NO and EDHF (Fig. 3) [24,25]. The existence of EDHF was first described in 1988 by Feletou and Vanhoutte et al. [26] and Chen et al. [27] independently and they are primarily found in resistance vessels, which are the principal regulators of vascular resistance and thus blood pressure. Thus, regulation of EDHF is the predominant mechanism for controlling vasodilation [28].

The contribution of H_2O_2 to EDHF-dependent vasodilation of resistance vessels [11–13] can be primarily attributed to the oxidation of protein kinase G, subunit 1α (PKG1α) [29]. Recent work from the Eaton Laboratory has demonstrated that PKG can be activated by an oxidation mechanism where the homodimer complex forms an interprotein disulfide [30]. This oxidation to the disulfide state is important for the catalytic activity of PKG. In endothelial cells, PKG activity is also regulated by intracellular cGMP levels, which can be modified by NO produced by shear stress and agonists, such as bradykinin, acetylcholine (ACh), and adenosine (Fig. 2) [31]. Furthermore, vascular smooth muscle expressing PKG resistant to H_2O_2 oxidation (i.e. Cys42 to Ser mutant) displays altered relaxation properties that may be due in part to changes in protein interactions with substrates such as RhoA, MYPT1 and BKCa channels [31] (Fig. 3). These effects were demonstrated using knock-in mice overexpressing Cys42Ser redox-dead PKG1α that fail to form a PKG disulfide and importantly those mice are hypertensive as compared with control mice [29,31].

The mechanism of H_2O_2-induced hyperpolarization seems to be complex and varies depending on the type of blood vessels tested. For example, Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ), caveolin-1 and PKG1α in murine microvessels have all been shown to play a substantial role in the enhanced EDHF-mediated response mechanism utilizing small amounts of O_2^- and H_2O_2 in vascular endothelial cells [32]. Bone marrow (BM) also appears to play an important role in modulating microvascular EDHF [33]. To address this notion, we transplanted BM from wild-type (WT) mice or $eNOS^{-/-}$ mice into male $eNOS^{-/-}$ mice and found that the reduced endothelium-dependent relaxations and hyperpolarizations of mesenteric arteries to ACh in $eNOS^{-/-}$ mice were markedly improved when transplanted with WT-BM but not with $eNOS^{-/-}$-BM [33]. Furthermore, the enhanced endothelium-dependent relaxations by WT-BM transplantation were abolished by catalase, indicating that the improved responses were mediated by H_2O_2.

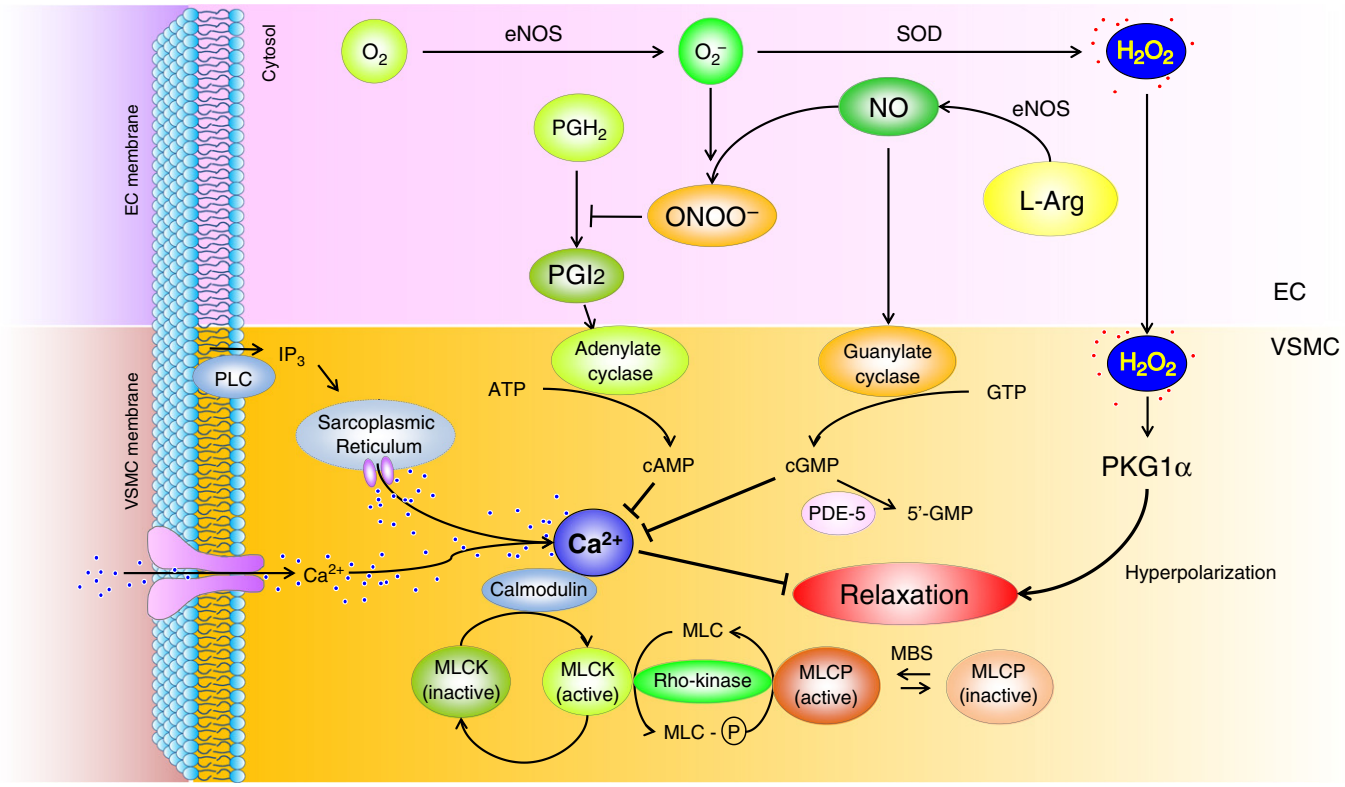

Fig. 3. Interaction between endothelial cells and vascular smooth muscle cells (VSMC) during vascular relaxation. The process involves multiple reactive oxygen species (ROS) and their targets as well as several proteins in the RhoA/Rho-kinase signaling pathway. EC, endothelial cells; PGI_2, prostacyclin; NO, nitric oxide; EDHF, endothelium-derived hyperpolarizing factors; NOS, nitric oxide synthase; PKG, protein kinase G; MLC, myosin light chain; MLCK, MLC kinase; MLCP, MLC phosphatase; MBS, myosin binding subunit.

The role of H_2O_2 as an EDHF has led to extensive research on the importance and complexity of endothelium-derived relaxing factors. Although our understanding of this vascular-derived oxidant is continually expanding, further studies are needed to clarify the physiological role of H_2O_2 in vascular homeostasis.

3.2. Pathological levels of ROS promote vascular disease

To date, most of the research on H_2O_2 has focused on its pathological roles. Diabetic vascular dysfunction is associated with an increase in ROS [34]. Cardiovascular diseases often result from imbalances in the levels of oxidative species in the cell. The O_2^--producing oxidases in the vascular system, including eNOS, cyclooxygenase, lipoxygenase, P-450 monooxygenase and NADPH oxidases [35], can be stimulated to produce excess ROS by external stimuli, such as mechanical stretch, pressure, shear stress, and hypoxia and by humoral factors such as angiotensin II [23,36]. Excessive ROS target multiple biomolecules, causing numerous cellular complications, including lipid peroxidation, protein oxidation/inactivation and DNA damage/mutations [23]. Furthermore, increased O_2^- levels attenuate endothelium-dependent relaxation and promote contraction in VSMC through formation of hydroxyl radicals [37,38] and can become H_2O_2 spontaneously or through SOD-dependent dismutation (Fig. 2). Although H_2O_2 is important for endothelial function and vascular relaxation at physiological low concentrations [11,13], pathological higher concentrations of ROS are hazardous to the cells, leading to endothelial dysfunction and VSMC proliferation. Furthermore, H_2O_2 is converted by endogenous peroxidases into either H_2O and O_2 or hydroxyl radicals, which are known to cause endothelium-dependent contractions through production of vasoconstrictor prostanoids in VSMC, leading to additional cellular damage [39].

Twenty years ago, very little was known about the proliferative response induced in VSMCs after arterial injury. ROS generated during arterial injury were considered, at least in part, to be responsible for this cellular response. Using xanthine/xanthine oxidase to generate ROS, Rao and Berk demonstrated that ROS stimulate VSMC proliferation in vitro and that H_2O_2 is the primary molecule responsible for xanthine/xanthine oxidase-induced VSMC DNA synthesis [40]. Both O_2^- and H_2O_2 stimulate VSMC growth, but only O_2^- rapidly activates MAP kinase, suggesting that additional signal events are involved in the mitogenic effects of H_2O_2 [9]. Based on these reports, excess amount of ROS appears to promote VSMC proliferation and increase the potential to develop vascular diseases. Interestingly, ROS stimulate extracellular-signal-regulated kinases 1 and 2 (ERK1/2) in a biphasic manner in VSMC, and one explanation for the delayed ERK1/2 activation is an involvement of the Secreted OXidative stress-induced Factors (SOXF) [41]. Cyclophilin A (CyPA) is one of the major proteins released into the medium in response to ROS [42]. Importantly, human recombinant CyPA stimulates ERK1/2 and DNA synthesis in VSMCs in a concentration-dependent manner [42]. Thus, extracellular CyPA is a novel growth factor that contributes to ROS-induced VSMC growth [43].

3.3. Potential limitations/drawbacks of therapeutically targeting either ROS or H_2O_2 in the vasculature

Exogenous H_2O_2 has been commonly used to elucidate the role of H_2O_2 in vascular function [14,44]. In the recent paper from the Gutterman laboratory, the EC50 of exogenous H_2O_2-induced dilation in human coronary arteries was approximately $1–3 \times 10^{-5}$ mol/L. This concentration is similar to those used in the other studies [44]. However, the concentrations used for the exogenous H_2O_2 application were higher than those reported for endogenously generated H_2O_2 ($<10^{-6}$ mol/L) in physiological conditions. The discrepancy of H_2O_2 concentrations between the exogenous stimulation and the endogenous generation would be explained in part by the fact that the limited

amount of exogenous H_2O_2 (1% to 15%) diffuses and reaches into the intracellular target components [14,44].

4. The role of CyPA in ROS-induced vascular disease

4.1. Function of CyPA

In 1984, intracellular CyPA was identified as the main target for the immunosuppressive drug, cyclosporine [45]. Cyclophilins are a family of highly conserved and ubiquitous proteins termed immunophilins [46]. The most abundant cyclophilin is CyPA, which is widely distributed in almost all tissues [47]. Owing to its enzymatic properties, cellular localization, and role in protein folding, CyPA is classified into a diverse set of proteins termed foldases [48]. It catalyzes the cis-trans isomerization of peptidyl-prolyl bonds of certain proteins (PPIase activity) and acts as an accelerant during protein folding and assembly. In addition to its role in protein folding, CyPA has recently been demonstrated to have a variety of functions, including intracellular trafficking, signal transduction and transcriptional regulation [49,50]. Importantly, CyPA plays a crucial role in the translocation of Nox enzymes such as p47phox [51], which are known to contribute to VSMC proliferation and development of vascular diseases [23]. Since ROS production by Nox enzymes activates other oxidase systems, CyPA and Nox enzymes amplify ROS formation in a synergistic manner, leading to increased oxidative stress.

It is now known that CyPA is secreted from the VSMC via a highly regulated pathway, which involves vesicle transport and plasma membrane binding in response to oxidative stress [52]. The expression of RhoA and Cdc42 (cell division control protein 42) dominant-negative mutants and a Rho-kinase inhibitor blocked ROS-induced CyPA secretion [52,53]. These results suggest that CyPA is secreted from VSMC through a process that requires ROS production, RhoA/Rho-kinase activation and vesicle formation [54]. In the VSMC, extracellular CyPA stimulates ERK1/2, Akt, and JAK (Janus protein tyrosine kinase) that contribute to ROS production [55,56] (Fig. 4). In endothelial cells, extracellular CyPA augments pro-inflammatory pathways, including enhanced expression of adhesion molecules, and promotes atherosclerosis [57,58]. In inflammatory cells, extracellular CyPA also works as a chemoattractant in cooperation with other cytokines and chemokines. Although the protein basigin has been proposed to serve as an extracellular receptor for CyPA in inflammatory cells [59], the identity of CyPA receptors in endothelial cells and VSMC remains unknown. Further knowledge of the extracellular CyPA receptors on vascular cells will contribute to the development of novel therapies for cardiovascular diseases.

4.2. CyPA promotes atherosclerosis

Changes in vascular redox state and extracellular CyPA are commonly involved in the pathogenesis of vascular restenosis [60], aortic aneurysms [53], atherosclerosis [58] and cardiac hypertrophy [61]. We demonstrated that CyPA (both intracellular and extracellular) contributes to atherosclerosis by promoting EC apoptosis and EC expression of leukocyte adhesion molecules. These actions stimulate inflammatory cell migration, enhance ROS production, increase proliferation of macrophages and VSMC and increase pro-inflammatory signal transduction in VSMC [58]. In the context of atherosclerosis, CyPA is regarded as a pro-inflammatory and pro-atherogenic molecule. The role of CyPA in inflammation was discovered using mice lacking both apolipoprotein E ($ApoE^{-/-}$) and CyPA ($CyPA^{-/-}$) that appeared to be protected from atherosclerosis development. The atheroprotection observed in $ApoE^{-/-}CyPA^{-/-}$ mice was due to the decreased levels of inflammation mediated by the absence of CyPA [62]. The vascular endothelium expresses a large array of vital proteins that function in normal cellular processes, the loss of which lead to initiation of atherosclerosis [63]. For example, eNOS function is critical for vascular homeostasis via generation of NO and its loss is pro-atherogenic. Furthermore, the progression of atherosclerosis is associated with decreases in both eNOS expression and NO production. In the $ApoE^{-/-}CyPA^{-/-}$ mice, aortic staining revealed significantly higher eNOS expression as compared with $ApoE^{-/-}$ mice [58], indicating that CyPA plays a role in regulating the eNOS/NO levels. Moreover, shear stress-induced eNOS expression was significantly increased when CyPA siRNA was used to silence CyPA in human umbilical vein endothelial cells (HUVEC) [58]. In addition, CyPA knockdown in HUVEC increased eNOS promoter activity and eNOS mRNA levels, whereas overexpression of CyPA reduced eNOS protein and mRNA levels. Both the antioxidants N-acetyl cysteine

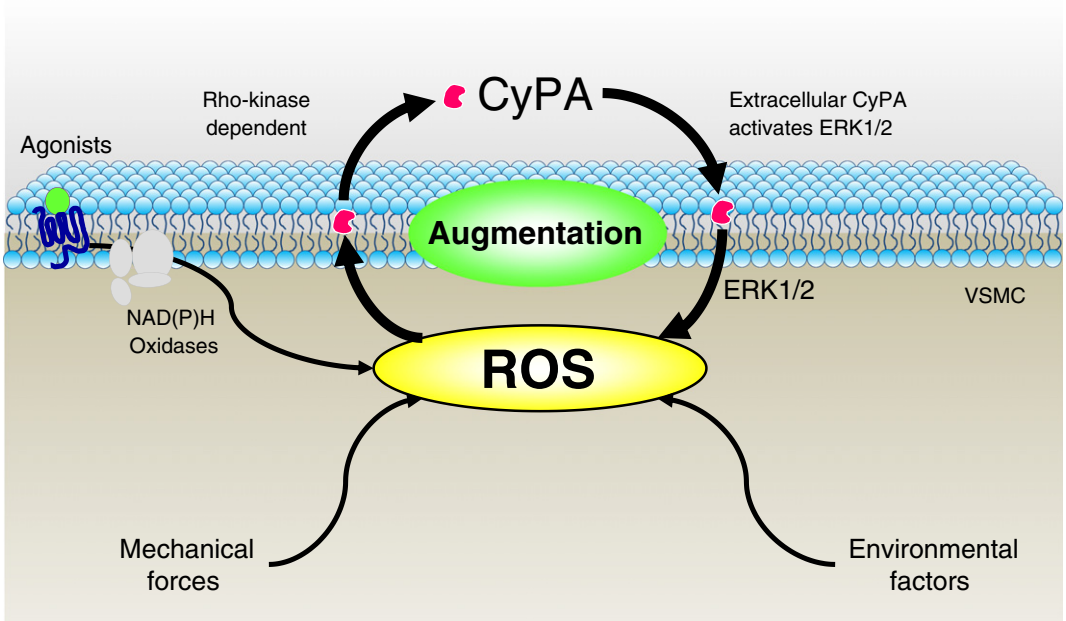

Fig. 4. Extracellular Cyclophilin A augments ROS production. Reactive oxygen species (ROS) inducer such as angiotensin II (AngII), mechanical stress, and environmental factors, promotes cyclophilin A (CyPA) secretion from vascular smooth muscle cells (VSMC). Secreted CyPA activates ERK1/2 and promotes ROS production, contributing to the augmentation of ROS production. ERK1/2, extracellular-signal-regulated kinase 1/2.

(NAC) and Tiron reversed this CyPA-mediated inhibition of eNOS promoter activity [58]. These findings suggest a novel mechanism by which CyPA promotes atherosclerosis through suppression of eNOS transcription.

Furthermore, overall ROS production is significantly higher in HUVEC overexpressing CyPA than in cells transfected with the vector control [58]. This suggests that CyPA plays a critical role in ROS generation in endothelial cells as in VSMC [64] and that CyPA likely induces inflammation through ROS-dependent mechanisms in those vascular cells [56]. Based on these results, CyPA is likely the primary mediator that augments ROS production, contributing to vascular inflammation and atherogenesis [56].

4.3. Clinical implications of oxidative stress research on CyPA

The identification of CyPA as a mediator of tissue damage associated with inflammation and oxidative stress provides new insight into the mechanisms of several therapies. We have recently demonstrated that plasma levels of CyPA are significantly elevated in patients with angiographically-proven coronary artery disease (CAD) [65]. Importantly, CyPA levels were also elevated in patients with hypertension, diabetes mellitus, smoking, dyslipidemia and advanced age [65], all of which are atherosclerotic risk factors as well as ROS-inducers. We also demonstrated that CyPA is a prognostic marker for cardiovascular intervention, such as percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG) [65]. Furthermore, after the treatment in several individuals, the plasma obtained at baseline and a follow-up revealed a significant reduction after the treatment [65]. Medical treatments that control atherosclerotic risk factors decreased plasma CyPA levels in CAD patients, suggesting that plasma CyPA is useful for the evaluation of systemic oxidative stress and the therapeutic effect by medication [65]. Taken together, these results suggest that circulating CyPA is a novel biomarker for CAD and plays a crucial and synergistic role in ROS augmentation, contributing to the progression of atherosclerosis [56]. Importantly, CyPA is highly expressed at sites with unstable atherosclerotic plaques, especially those associated with macrophages and foam cells [65]. However, CyPA expression during plaque destabilization in humans and its regulatory mechanism still remain elusive. Further research regarding the role of CyPA in the progression of atherosclerosis is necessary to identify potential CyPA-related therapeutic targets.

5. Future perspectives

Numerous basic and clinical studies have demonstrated that ROS play a major role in the pathogenesis of endothelial dysfunction and atherosclerosis. However, no therapeutic strategies are yet available for clinical use of the antioxidants. We consider that one of the reasons for this dilemma is that low concentrations of ROS, particularly H_2O_2, play an important role in intracellular signaling pathways that are crucial for numerous vascular cell functions. The source/location of ROS production may also be important in determining their physiological or pathological roles. For example, the roles of ROS in endothelial cells, VSMC and migrating perivascular inflammatory cells would be different between physiological and pathological conditions. In addition, the dual roles of ROS may be somewhat analogous to the beneficial/deleterious actions of NO in cell signaling [20]. Furthermore, the production of ROS in inflammatory cells plays a crucial role in the cellular responses in immune response and infection [66]. Although many strategies to control oxidative stress have been previously tested in various diseases, we need to pay close attention to the existence of a complex network of molecules that exogenously or endogenously contribute to the balance between oxidant and antioxidant systems. Thus, it may be an important clinical strategy to use antioxidants and/or drugs that can prevent oxidation of selected redox-sensitive targets under certain disease conditions, while allowing the ROS to continue to function in normal processes.

Furthermore, the identification of CyPA as a mediator of oxidative stress-induced tissue damage has provided some additional insight into the mechanisms of several therapies. For example, Rho-kinase inhibitor and simvastatin significantly reduced CyPA secretion from VSMC [52,64]. Indeed, Rho-kinase is an important therapeutic target in cardiovascular diseases [67] and Rho-kinase inhibition has been reported to reduce AngII-induced abdominal aortic aneurysm (AAA) formation [68], atherosclerosis, pulmonary hypertension [69] and cardiac hypertrophy [70]. Moreover, angiotensin II type 1 (AT1) receptor blockers and angiotensin-converting enzyme (ACE) inhibitors have been shown to reduce cardiovascular diseases [71–73], for which reduced CyPA secretion may be involved as shown in AAA, atherosclerosis and pulmonary hypertension [64].

Based on the role of extracellular CyPA, it is logical to consider that agents that prevent CyPA receptor binding and reduce circulating CyPA may have therapeutic potentials. Blocking this vicious cycle that augments ROS production through CyPA autocrine/paracrine signaling pathway could be a novel therapeutic tool for controlling cardiovascular diseases. However, the regulation of CyPA expression and the identity of its extracellular receptors remain largely unknown. Thus, further basic and clinical studies are needed to identify CyPA-related therapeutic targets in the future [74].

Disclosure statement

None.

Acknowledgments

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