Review article

Redox signaling in cardiovascular pathophysiology: A focus on hydrogen peroxide and vascular smooth muscle cells

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A B S T R A C T

Oxidative stress represents excessive intracellular levels of reactive oxygen species (ROS), which plays a major role in the pathogenesis of cardiovascular disease. Besides having a critical impact on the development and progression of vascular pathologies including atherosclerosis and diabetic vasculopathy, oxidative stress also regulates physiological signaling processes. As a cell permeable ROS generated by cellular metabolism involved in intracellular signaling, hydrogen peroxide (H_2O_2) exerts tremendous impact on cardiovascular pathophysiology. Under pathological conditions, increased oxidase activities and/or impaired antioxidant systems results in uncontrolled production of ROS. In a pro-oxidant environment, vascular smooth muscle cells (VSMC) undergo phenotypic changes which can lead to the development of vascular dysfunction such as vascular inflammation and calcification. Investigations are ongoing to elucidate the mechanisms for cardiovascular disorders induced by oxidative stress. This review mainly focuses on the role of H_2O_2 in regulating physiological and pathological signals in VSMC.

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Contents

1. Introduction

Oxidative signaling is critical for cell homeostasis and survival. Reactive oxygen species (ROS) are the small molecules responsible for this signaling, and they are produced at low levels continually during normal cell function. In particular, hydrogen peroxide (H_2O_2) has become recognized as a crucial mediator of cellular oxidative signaling [1,2]. The physiological level of H_2O_2 is maintained in the cell by a series of enzymatic actions, including NADPH oxidases (NOX) and superoxide dismutases (SOD), through the dismutation of superoxide anion ($O_2^{\bullet-}$) [3,4]. H_2O_2 is essential in signaling pathways determining cell viability, and it participates in the cell's ability to combat bacteria and other pathogens [5]. Under pathological conditions, such as hypertension, diabetes, and hyperlipidemia – the major risk factors of atherosclerosis, however, elevation of vascular NOX increased H_2O_2 production, which leads to monocyte/macrophage infiltration, lipid oxidation, foam cell formation that significantly contribute to vascular inflammation and lesion development [6–8].

Recent studies by our group and others have elucidated a mechanistic link between H_2O_2-induced oxidative stress and vascular cell homeostasis and differentiation [9,10]. We have shown that H_2O_2 induces phenotypic changes of vascular smooth muscle cells (VSMC) that lead to vascular pathologies such as calcification [9]. This review aims to highlight the role of H_2O_2 as a physiological and pathological redox signal in VSMC, as well as newly discovered molecular signals promoting oxidative stress in cardiovascular diseases.

2. Regulation of H_2O_2 production in VSMC

ROS can be generated following cell stimulation and function as intracellular signaling molecules [11,12], and oxidative species have been shown to be critical for cell homeostasis and survival [13]. Excessive ROS generated by cellular metabolism, however, causes cellular damage and tissue dysfunction [14]. H_2O_2, a cell permeable ROS that can diffuse across biological membranes and has a relatively long half-life among other ROS [15], has been shown to serve as an effective redox signaling mediator that regulates intracellular signaling [16,17]. H_2O_2 is produced in vascular cells by multiple enzymatic systems [18]. While mitochondria are responsible for the majority of H_2O_2 production within the cell under physiological conditions, some non-mitochondrial sources of H_2O_2 have also been described, including vascular NOX, xanthine oxidase and uncoupled eNOS [19,20]. Under normal conditions, constitutive oxidase activities and endogenous scavenger systems, including catalase and glutathione peroxidases, maintain steady-state H_2O_2 levels in vascular tissue [20,21]. H_2O_2 is important to maintain VSMC function under physiological conditions, such as proliferation, migration, and differentiation [22,23]. Upon stimulation, multiple oxidase systems in the endothelium, media, and adventitia can produce H_2O_2 and contribute to elevation of H_2O_2 in VSMC [24,25]. Exogenous H_2O_2 can further induce production of endogenous H_2O_2 by activating cellular NOX [3]. In this way, chronically accumulated H_2O_2 leads to VSMC dysfunction and damage in the vasculature [26].

2.1. Mitochondrial H_2O_2 production

The precursor of H_2O_2, superoxide anion ($O_2^{\bullet-}$), is produced mainly by complexes I and III in the mitochondrial respiratory chain [27,28]. Superoxide anion generated in the intermembrane space can be carried across the membrane to the cytosol via anion channels [29]. The presence of mitochondrial SOD-2, which consumes superoxide anion as it is produced and converts it to H_2O_2,

and other similar enzymes, protects the mitochondrion itself from the damaging effects of ROS [30]. Several studies have described the production of superoxide anion by the mitochondrial respiratory complexes [31–33]. The contributions of each of the complexes and individual enzymes to the overall superoxide anion production vary depending on the organ and the reduced state of the complexes. Also the capacity of each site relies on the abundance of the enzymes or complex in the mitochondria. I_Q in Complex I has been determined to be the predominant center of production for superoxide anion and/or H_2O_2 in the mitochondria during reverse electron transport [34]. Among eleven sites of superoxide anion and/or H_2O_2 production in mitochondria, most of the sites release superoxide anion and H_2O_2 exclusively in the mitochondrial matrix due to their location in the matrix or at the inner face of the matrix. While site III_{Qo} in Complex III and G_Q linked to the mitochondrial glycerol 3-phosphate dehydrogenase generate superoxide anion and/or H_2O_2 to the external side of the mitochondrial inner membrane and the matrix. The topological differences in the release of superoxide anion and H_2O_2 to different compartments in the mitochondria are likely to have significant impact on downstream redox signaling [34]. To better understand the role of mitochondrial ROS in oxidative damage and redox signaling in the vasculature, state-of-the-art methods to measure mitochondrial superoxide anion and H_2O_2 formation in vivo are definitely needed as the generation within the mitochondrial matrix highly depends on local oxygen concentration and redox groups bound to relevant protein, which are all highly variable and difficult to measure in vivo.

Non-mitochondrial H_2O_2 Production.

Many non-mitochondrial enzymes have been shown to produce H_2O_2 [35,36]. Xanthine oxidase produces H_2O_2 as an intermediate for the reaction in which xanthine is converted to uric acid [37,38]. Lipoxygenase produces oxidized linoleic acid and cyclooxygenase produces prostaglandins from arachidonic acid, each of which results in H_2O_2 production [39]. These systems are known to participate in the redox signaling under physiological conditions, while causing toxic cellular effects and diseases once their activity is dysfunctional.

On the other hand, NOX, a multi-enzyme complex, produces H_2O_2 or superoxide anion primarily, not as a part of other reactions [35,40]. NOX exists in many different isoforms, all using NADPH as a donor of the electron to generate H_2O_2 or superoxide anion [41]. In VSMC, Nox1, Nox2, and Nox4 have been linked to multiple cardiovascular diseases, including hypertension, atherosclerosis, and diabetic vasculopathy [42–45]. Nox1 is primarily found in caveolae, endosomes, and plasma membranes of VSMC [46] and it has a critical role in VSMC proliferation in response to angiotensin II (Ang II), PDGF, and thrombin [47–49]. Nox1-deficiency inhibited injury-induced neointimal formation, which was associated with inhibition of VSMC proliferation and migration [50]. In contrast, enhanced activation of Nox1 by increased NoxA1, an activator of Nox1, in the vasculature increased superoxide anion generation in VSMC and increased neointimal hyperplasia and atherosclerosis [49]. Similarly, increased Nox1 expression in cultured VSMC or aortic segments induced VSMC proliferation [51]. Although the precise molecular mechanisms underlying Nox1-mediated proliferation of VSMC are not well defined, activation of a small GTP-binding protein ADP-ribosylation factor 6 and MAPK signaling pathways are demonstrated in Ang II-induced VSMC proliferation [47]. Nox2 is expressed in endosomal and phagosomal membranes and is regulated in a similar way to vascular Nox1 [52]. Nox2 has also been described as an important regulator of fibroblast proliferation since its downregulation results in reduction of serum-induced proliferation [122,123]. Recent report demonstrates that ROS-derived from Nox2, an important regulator of fibroblast proliferation act as a paracrine stimulus on neointimal hyperplasia

and medial smooth muscle hypertrophy [53]. In addition, Nox4 has been shown to play an important role in focal adhesions and maintenance of differentiated status of VSMC [54]. A recent study suggests that Nox4 is critical for transforming growth factor beta-regulated expression of VSMC differentiation marker, such as smooth muscle α-actin, by activation of a p38 and serum-response factor/myocardin-related transcription factor pathway NOX-generated H_2O_2 in VSMC has been shown to be extensively involved in hypertrophy, proliferation, migration, and inflammation [55]. Therefore, the NOX enzymes have become recognized as highly important targets in the development of therapeutic strategies for ROS production in physiological and pathological conditions.

2.2. Physiological roles of H_2O_2 in VSMC

Although high levels of ROS cause cellular dysfunction and damage in the vasculature, normal levels of ROS are important to maintain physiological responses. In particular, H_2O_2 plays an important role in cellular signaling in a range of biological processes in many organisms. Due to its ability to travel through membranes, H_2O_2 is involved in signaling in every cellular organelle. H_2O_2 has been well recognized as a critical mediator of VSMC function under physiological conditions, such as proliferation, migration, and differentiation as well as modulation of vascular tone.

As H_2O_2 activates some common signaling molecules and pathways induced by growth factors, it has long been regarded as an early growth signal. Brown et al. showed in an early study that H_2O_2 promotes VSMC proliferation [56]. They found that overexpression of catalase, the enzyme that hydrolyzes H_2O_2, reduces H_2O_2 production in rat VSMC and inhibits cell growth, suggesting that endogenously produced- H_2O_2 at physiological levels may play a fundamental role in VSMC proliferation. On the other hand, exogenous addition of H_2O_2 (10 nM to 100 μM) to growth-arrested human VSMC was found to stimulate an increase in cell growth, which was antagonized by catalase in a dose-dependent manner [57]. Similarly, in growth-arrested rabbit VSMC, H_2O_2 synergistically enhanced angiotensin II-induced VSMC proliferation [58]. In human coronary artery smooth muscle cells, H_2O_2 increased the half-life and induced the expression of placenta growth factor [59], an important mediator of arteriogenesis [60,61] that regulates angiotensin II-induced VSMC proliferation [62]. Collectively, these results support the notion that H_2O_2 is a critical regulator of VSMC proliferation under physiological conditions.

Migration of VSMC is an important process in the development of blood vessels as well as vascular pathology, including neointimal formation and atherosclerosis. Platelet-derived growth factor (PDGF) is one of the most potent migratory factors for VSMC. PDGF-induced migration of VSMC was attenuated by pre-treatment with antioxidants including N-acetyl-cysteine, the glutathione peroxidase (GPx) mimetic, ebselen, as well as catalase, the scavengers of H_2O_2, indicating an important role of H_2O_2 as a cellular mediator in PDGF-induced VSMC migration [63]. In addition, insulin and insulin-like growth factor 1 signals have been shown to play important roles in vascular remodeling. Similar to exogenously added H_2O_2, H_2O_2-induced by insulin/insulin-like growth factor 1 was found to elevate motility of rat VSMC, which was blocked by catalase [64]. Recently, an elegant study by de Rezende et al. showed that H_2O_2 oxidizes specific cysteine residues within the α7 subunit of integrin α7β1, which facilitates VSMC formation of membrane protrusions via binding to laminin-111, thereby enhancing the binding affinity of integrin to laminin that increases VSMC migration [65].

In addition to its roles in regulating VSMC physiological responses such as proliferation and migration, H_2O_2 has also been demonstrated to regulate differentiation of VSMC from their precursors as well as phenotypic changes between "contractile" state and "synthetic" state. Xiao et al. found that a set of SMC-specific genes was significantly upregulated in mouse embryonic stem cells cultured on collagen IV-coated plates, which was correlated with an increased expression of NOX4, the H_2O_2 producing enzyme [66]. They further demonstrated that NOX4 induced-H_2O_2 mediated the differentiation of stem cells into VSMC via activation of SMC-specific transcription factors, including serum response factor and myocardin. In contrast, inhibition of NOX4 activity due to the deficiency of polymerase delta interacting protein 2 reduced H_2O_2 production in VSMC, which resulted in an elevated secretion of collagen I in VSMC and led to increased stiffness, reduced compliance, and excessive extracellular matrix deposition in mice, implicating phenotypic changes of VSMC into synthetic forms under reduced H_2O_2 level [67]. On the other hand, exogenous addition of H_2O_2 was found to directly induce the expression of microRNA-145 in rat VSMC, which increased the expression and activity of the key VSMC transcriptional factor, myocardin [68]. Consistently, increased H_2O_2 production in VSMC from p22phox-over-expressing mice exhibited increased expression of synthetic phenotypic markers concomitantly with decreased contractile markers, supporting the regulation of H_2O_2 production on VSMC phenotypic modulation [69].

In the vascular walls, endothelial cells modulate underlying VSMC and vascular tone by releasing relaxing and contracting factors, including nitric oxide and H_2O_2 [70,71]. In human coronary arterioles, shear stress induces endothelial cell-derived H_2O_2 that results smooth muscle hyperpolarization and relaxation, which could be abrogated by polyethylene glycol catalase [71]. In rat renal artery, acetylcholine triggers transient smooth muscle contraction in an endothelium-dependent manner [72]. Inhibition of NOX, however, attenuates acetylcholine-induced smooth muscle contraction, suggesting that acetylcholine stimulates NOX-derived H_2O_2 production in endothelial cells that leads to smooth muscle contraction [72]. Therefore, endothelial production of H_2O_2 may act as endothelium-derived hyperpolarization factor that regulates VSMC function and vascular tone.

2.3. Scavengers of H_2O_2

Under normal conditions, the level of H_2O_2 is maintained in steady-state in the vasculature by constitutive oxidase activities and endogenous scavenger systems, including the major families of antioxidant enzymes: SOD, catalase, glutathione peroxidases, and thioredoxin peroxidases. These antioxidant enzymes play important roles as molecular sensors and biological modulators of the effects exerted by ROS on cellular signaling events.

There are three SOD isoforms in human: cytoplasmic SOD1 (Cu-Zn), mitochondrial SOD2 (Mn), and extracellular SOD3 (Cu-Zn). SOD1 knockout mice exhibit increased superoxide anion while reduced cytoplasmic H_2O_2 in pulmonary arteries, resulting in an increase in the ratio of superoxide anion H_2O_2 in pulmonary arterial SMC that led to vascular inflammation and pulmonary hypertension [73]. Accordingly, fine-tuning the balance among ROS by anti-oxidant enzymes such as SODs regulates cellular oxidative signaling pathways in VSMC.

Catalase acts more directly to decompose H_2O_2 into water and oxygen; its importance is reflected by its ubiquity in all oxygen-consuming organisms. The expression and activity of catalase in aortic wall were closely correlated with the formation of abdominal aortic aneurysms (AAA) in mice. It was shown that the administration of PEG-catalase prevents the loss of tunica media and the formation of AAA induced by calcium chloride on mouse infrarenal aortas [74]. Similarly, over-expression of catalase in VSMC results in enhanced VSMC survival and reduced AAA formation through restored catalase activity and decreased matrix
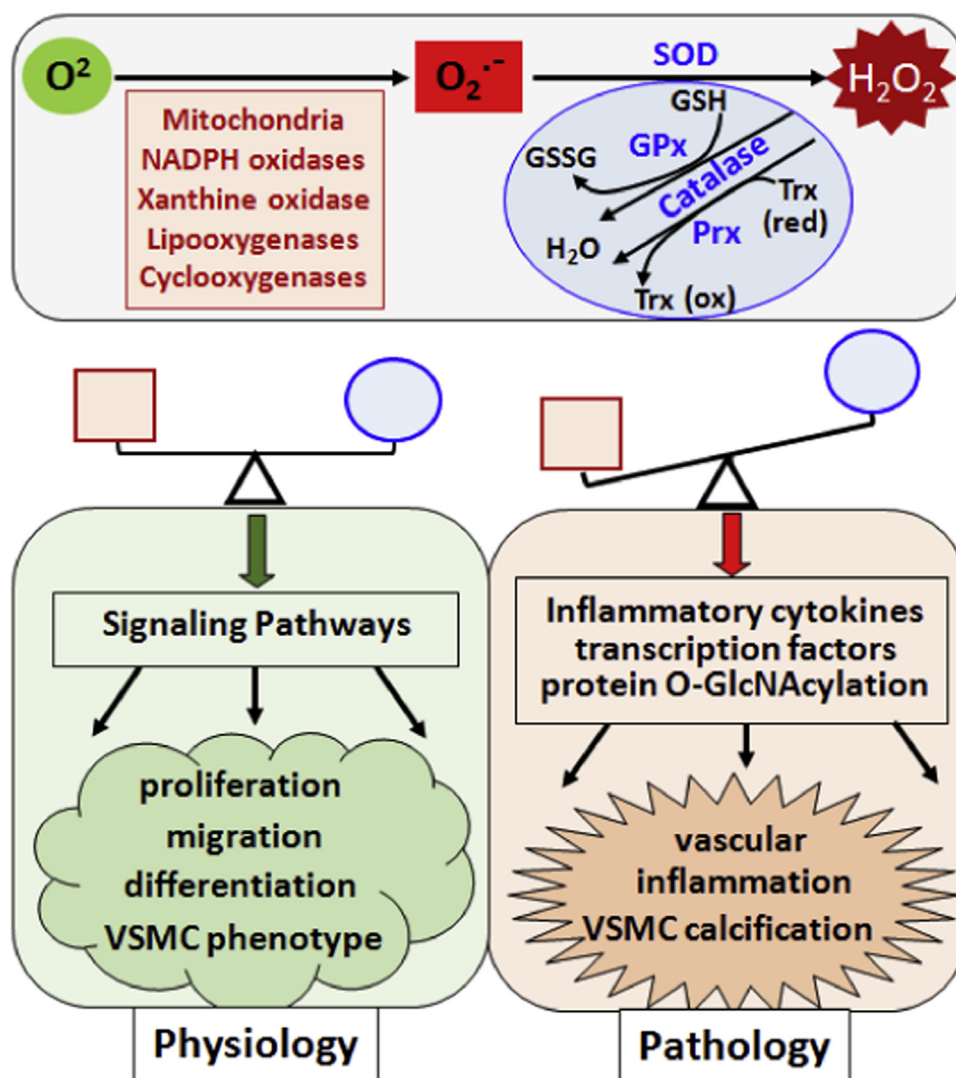
Fig. 1. Regulation and function of hydrogen peroxide in modulating VSMC physiology and vascular dysfunction, such as vascular inflammation and calcification. Hydrogen peroxide (H_2O_2) is produced in vascular smooth muscle cells (VSMC) through mitochondrial respiratory chain and non-mitochondrial enzymatic systems via superoxide anion ($O_2^{\bullet-}$), converted to H_2O_2 by superoxide dismutases (SOD). One of the major families of antioxidant enzymes, catalase directly catalyzes H_2O_2 into water. Glutathione peroxidases (GPx) and peroxiredoxins (Prx) scavenge H_2O_2 by the use of reducing power of glutathione (GSH) and thioredoxin (Trx). Under physiological conditions, homeostasis of cellular redox status is maintained by keeping the steady-state levels of H_2O_2 through the balance between oxidase systems and antioxidant machinery, allowing H_2O_2 to serve as a critical modulator of cellular signaling events. Excessive accumulation of H_2O_2 by increased oxidase activities and/or impaired antioxidant systems, however, leads to the pathological conditions, such as increased vascular inflammatory responses and VSMC calcification. GSH, reduced glutathione; GSSG, oxidized glutathione; Trx (red), reduced thioredoxin; Trx (ox), oxidized thioredoxin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

metalloproteinase activity [74]. In addition, the over-expression of human catalase in the atherogenic ApoE$^{-/-}$ mice contributes to the retarded atherosclerotic development correlated with the reduced F2-isoprostanes in the plasma and aortas, suggesting that H_2O_2 is implicated in the formation of oxidized lipids and the development of atherosclerosis in ApoE$^{-/-}$ mice [75].

More recently, studies with the use of new animal models have demonstrated the importance of the glutathione peroxidase (GPx) antioxidant systems in maintaining vascular redox balance. Eight different isoforms of GPx have been currently identified in humans and each functions as a reducing molecule of H_2O_2 to water. GPx1 is the predominant glutathione peroxidase isoform in the vasculature. VSMC from GPx1-haplodeficient mice demonstrated increased oxidative stress that led to NF-κB activation, VSMC proliferation, and an inflammatory response [76]. Global knockout of GPx1 in ApoE$^{-/-}$ mice resulted in increased oxidative stress and accelerated development of atherosclerotic lesions [77], supporting a direct role of GPx1 in regulating the development of vascular disease.

In addition, the thioredoxin system in vascular cells has been recognized as a critical anti-oxidizing entity and seems to mostly depend on thioredoxin peroxidase (peroxiredoxin, Prx) for its ROS-scavenging activities. Over-expression of Prx4 led to the attenuation of atherosclerotic development in ApoE$^{-/-}$ mice through the suppression of oxidative damage indicated by reduced markers of oxidative stress (8-hydroxy-2'-deoxyguanosine and oxLDL) and down-regulation of apoptosis [78]. Similarly, the deficiency in Prx2 results in increased production of H_2O_2 through enhanced activation of PDGF signaling and subsequent cell proliferation, while the forced expression of Prx2 in VSMC attenuates PDGF-induced activation of the PDGFR-β, possibly through inhibition of PDGF-induced H_2O_2 production [79]. Accordingly, Prx 2/4 might affect H_2O_2-induced mitogenic and migratory signaling, thus contributing to PDGF-induced VSMC proliferation and migration during neointimal formation [78,79].

New findings at the subcellular level have furthered our understanding of redox balance in the vasculature. Cellular

movement and location of antioxidant enzymes provides an efficient system of control for H_2O_2 at its site of generation. Activities of GPx or thioredoxin systems have been observed in several distinct subcellular locations, depending on the isoforms and the cellular environment. In mammals, GPx1 is found in the cytoplasm while GPx2 and GPx3 are mainly expressed extracellularly [80]. Mammalian cells also express two thioredoxin systems: the cytosolic thioredoxin 1 and the mitochondrial thioredoxin 2 [81]. Therefore, ROS, including H_2O_2, may be appropriately monitored and regulated by distinctly located antioxidant systems both intracellularly and extracellularly. Such a notion is supported by recent studies with targeted expression of the antioxidant enzymes in specific subcellular compartments. For instance, expression of Prx 5 in the mitochondrial intermembrane space was found to inhibit hypoxia-induced oxidative signaling in the mitochondrial intermembrane space as well as the cytosol in pulmonary arterial SMC [82]. Additionally, expression of a mitochondria-exclusive variant form of GPx1 resulted in a higher level of oxidative stress compared to cells expressing GPx1 mostly in the cytoplasm where the natural counterpart of GPx1 [83], further demonstrating the importance of the subcellular localization of the antioxidant enzymes.

2.4. H_2O_2-regulated signaling pathways

Hydrogen peroxide is a relatively weak oxidizing agent; however, it could reversibly and covalently induce post-translational modifications of cysteine thiolate residues resulting in changes in activity and function of target proteins [84]. H_2O_2 sensors exist throughout the cell to maintain cellular homeostasis. A variety of protein kinases, which bear a redox-sensitive cysteine residues in the kinases themselves or in their upstream signaling molecules, are involved in the alteration of VSMC function by catalyzing the phosphorylation of their target proteins. H_2O_2 has been implicated in the activation of c-Src, protein kinase C (PKC), phosphatidyl inositol (PI)3-kinase, protein kinase B (AKT), extracellular-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and mitogen-activated protein kinases (MAPK) [9,85–88]. H_2O_2 can induce downstream signaling by both autocrine and paracrine mechanisms. Recent studies show that H_2O_2 may be transported through membrane pores or by matrix vesicles [15,89], but this movement has yet to be well characterized.

The redox-sensitive protein kinases, MAPKs, are key regulators of signaling pathways that govern diverse cellular responses such as proliferation, differentiation, growth, and inflammatory responses [90]. Although MAPK are not known to be directly redox signaling sensitive, they experience redox regulation by ROS-activated upstream molecules such as src-family kinases and PKC [91,92]. Enhanced phosphorylation of ERK and p38 MAPK by H_2O_2 is observed in VSMC and endothelial cells, reportedly through the activation of an upstream src-family kinases and PKC, which results in the altered vascular function such as VSMC constriction [93,94]. ROS-dependent phosphorylation on Thr423 of p21-activated protein kinase has also been shown to regulate VSMC migration induced by PDGF [63]. Emerging evidence has connected ROS to PI3K signaling. PI3K signaling is activated in response to the exogenous H_2O_2 in various cell types [95]. H_2O_2 induces NADPH oxidase-mediated ROS which is dependent on PI3K/AKT signaling [96]. Thus, ROS activate PI3K, which in turn promotes the process leading to ROS accumulation. H_2O_2 has been shown to induce tyrosine phosphorylation of p110 (PI3K subunit) and enhances PI3K membrane recruitment to its substrate site, thereby enabling PI3K to maximize its catalytic efficiency [97]. H_2O_2-activated PI3K can lead to activation of AKT [98]. AKT acts as a transducer of PI3K signaling initiated by many stimuli and activates multiple cellular signaling pathways that regulates proliferation, differentiation, and survival [9,99,100].

On the other hand, protein tyrosine phosphatases (PTP) are directly inhibited by ROS via thiol modifications of cysteine residues in their catalytic sites [101]. The tumor suppressor phosphatase and tensin homolog (PTEN), which antagonizes the signaling cascade regulated by PI3K/AKT, is oxidatively inactivated by mitochondrial NOX-produced H_2O_2 [95]. In SOD2-overexpressing endothelial cells, increased mitochondrial H_2O_2 increases the ratio of inactive/active PTEN, which results in enhanced PI3K/AKT signaling that induces the expression of the key regulator of angiogenesis, vascular endothelial growth factor (VEGF) [102]. Further studies are warranted to better understand the complex system of H_2O_2-mediated regulation of kinases and phosphatases in VSMC.

Downstream of a number of the signaling cascades, ROS-sensitive transcription factors determine gene expression in response to changes in redox balance. NF-κB is a critical transcription factor for the proliferation, survival, and inflammatory responses in VSMC. Enhanced H_2O_2 in GPx1-deficient VSMC induces activation of NF-κB in quiescent VSMC, and thus leading to VSMC proliferation [76]. The nuclear factor erythroid 2-related factor 2 (Nrf2), a redox sensitive transcription factor, has also been shown to increase the expression of antioxidant enzymes that protect VSMC against oxidative stress [105,106]. Under low oxidative stress, Nrf2 is sequestered by Kelch-like ECH Associated Protein 1 and targeted for proteasomal degradation [107]. In high oxidative stress conditions, such as atherosclerotic and diabetes, activation of Nrf2-regulated antioxidant systems has been demonstrated in vascular endothelial cells and VSMC [108]. In addition, Furthermore, H_2O_2 has also been shown to induce phosphorylation of the cyclic AMP response element–binding protein (CREB), a transcription factor that is known to play an important role in neuron cell survival [103]. In contrast, hypoxia increases intracellular H_2O_2 via increased expression of NOX in pulmonary arterial smooth muscle cells (PASMC), thus inhibiting CREB and increasing PASMC proliferation [104]. The cell type-dependent differential regulation of CREB by H_2O_2 indicates the fine-tuning of cellular H_2O_2 signaling.

2.5. Pathological effects of hydrogen peroxide on vascular smooth muscle cells

Excessive generation of ROS induced-oxidative stress is a predominant factor that accelerates the progression of cardiovascular diseases, including atherosclerosis and vascular calcification [109–111]. Multiple risk factors associated with pathogenesis of atherosclerosis including hypercholesterolemia, diabetes, hypertension, smoking, and family history of heart attacks are all unequivocally linked to increased oxidative stress in the vasculature [112]. Pathological impact exerted by H_2O_2 on VSMC most relevant to the onset and aggravation of the disease will be further discussed.

2.6. H_2O_2 induces VSMC inflammation

Atherosclerosis has been well characterized as a prolonged inflammatory state within the cardiovascular system, in which a variety of cell types, including VSMC, endothelial cells, macrophages, and immune cells interact with each other and elicit potent inflammatory reactions in response to a variety of cellular stimuli. Recruitment and infiltration of blood-borne monocytes/macrophages into the arterial wall plays an important role in the development of atherosclerosis [113,114]. The infiltrated inflammatory cells produce tumor necrosis factor-alpha (TNF-α), a major pro-inflammatory cytokine in the development of atherosclerosis, which promotes the generation of ROS such as H_2O_2 via NOX- or mitochondria-dependent pathways [115–117]. Local H_2O_2 production from both vascular cells and infiltrated cells initiates a signaling cascade which leads to the inflammatory response through the expression of inflammatory cytokines and adhesion

molecules [118,119]. The critical role of inflammatory responses in VSMC during the development of atherosclerosis has also been well documented. In response to TNF-α, NF-κB signaling is activated in VSMC, which leads to increased expression of pro-inflammatory molecules such as vascular cell adhesion molecule-1, monocyte chemoattractant protein-1 (MCP-1), and fractalkine [120]. In rat VSMC, H_2O_2 treatment induces an increase in intracellular and extracellular osteopontin, an important mediator of inflammation and generation of atherosclerotic lesions [121]. In contrast, transgenic mice overexpressing VSMC-specific catalase, the enzyme that hydrolyzes H_2O_2, exhibit significant reduction in inflammatory molecules in the vessel walls, including TNF-α along with other inflammatory markers such as TGF-β, osteopontin, IL-1β, and MCP-1 [74]. These observations support a critical role of H_2O_2 produced by VSMC in regulating vascular inflammation. Furthermore, lowered macrophage infiltration into the arterial wall is observed in these catalase-overexpressing mice [74]. Therefore, VSMC-produced H_2O_2 may contribute to the overall progression of atherosclerosis by regulating the expression of inflammatory molecules in the vascular cells as well as the infiltration of inflammatory cells. In addition, studies from our group have demonstrated that H_2O_2 induces the expression of the receptor activator of nuclear factor kappa-B ligand (RANKL) in VSMC, which promotes macrophage infiltration [122,123]. The expression of RANKL is low in normal arteries, but increased in atherosclerosis and vascular calcification [124]. Our finding has supported a novel role of H_2O_2-stimulated RANKL elevation by VSMC in promoting macrophage infiltration in the pathogenesis of atherosclerosis. As indicated above, the infiltrated monocytes/macrophages induce production of inflammatory molecules as well as H_2O_2 from the activated inflammatory cells, forming a vicious cycle of H_2O_2 and inflammatory cytokine production in the vasculature.

2.7. H_2O_2 induces VSMC calcification

One of the characteristic features of atherosclerosis is the development of vascular calcification, which reduces the elasticity and decreases the compliance of vessel walls [125]. Although the presence of vascular calcification has long been recognized, we have only begun to understand the underlying mechanisms that regulate the development of vascular calcification in the last two decades. It is now well accepted that vascular calcification is not simply a passive precipitation of hydroxyapatite crystals but a regulated process that vascular cells differentiate into bone-like cells, a process resembling embryonic osteogenesis [126,127].

We and others have shown that oxidative stress is a major cause of vascular calcification [9,128–130]. Increased oxidative stress has been well documented in human atherosclerotic lesions. Studies by Terentis et al. have demonstrated oxidation of tocopherol along with LDL oxidation early in lesion formation [131]. Several studies have proven that oxidative stress signaling is pathologically increased in many cell types involved in atherosclerosis, including platelets [132], endothelial cells [133], macrophages [134], and VSMC [129]. We have reported that increased oxidative stress is associated with vascular calcification in atherogenic ApoE$^{-/-}$ mice [122,123]. Increased oxidative stress induces the expression of Runx2, a protein normally expressed in osteoblasts [135]. The critical role of Runx2 in vascular calcification is highlighted using mice with smooth muscle cell-specific genetic deletion of Runx2, which exhibit decreased vascular calcification and even show attenuation in overall atherosclerosis [122]. In vitro studies with human VSMC further show that many of the components causing atherosclerosis such as cholesterol and hyperglycemia, increase oxidative stress and associated signaling [136,137]. Sustained elevation in oxidative stress ultimately results

in atherosclerosis. The observation that oxidant generation is predominant near the calcifying foci [129] suggests the regulation of vascular calcification by redox signaling. Increased ROS, particularly H_2O_2, found around calcifying foci, may be attributed to enhanced expression of the oxidases, including Nox2, p22phox, and Nox4 [129], or may be due to reduced antioxidant systems such as catalase and SOD in calcified aortic valve [130].

Increased oxidative stress is also manifested in diabetic subjects [138] and in the vasculature of diabetic animals [139], where vascular calcification is predominantly identified in the media of VSMC. Specific mechanisms may be responsible for the oxidative stress observed in diabetes, independent of atherosclerosis or other cardiovascular diseases. Recent studies have shown that advanced glycation end products (AGEs) cause VSMC calcification in vitro through increased oxidative stress [139,140]. Diabetic patients and murine models exhibit increased expression of AGEs, which bind to the receptor for advanced glycation to increase oxidative stress [141,142]. This is a non-enzymatic mechanism of oxidative stress generation, caused by an excessive glucose which is characteristic of diabetes [143]. The levels of AGEs are elevated in diabetic patients as well, and are correlated with increased vascular calcification in these patients [144]. According to previous in vitro studies, the oxidative stress produced by the AGEs may be the causative factor in diabetic vascular calcification in diabetic patients [139,140,142].

In vitro studies have also shown that AGEs, which accumulate in diabetic patients, induce VSMC calcification, via increased oxidative stress by Nox4 and p22phox-dependent signals [140]. Our recent studies have highlighted the novel function of protein O-linked β-N-acetylglucosamine modification (O-GlcNAcylation) in regulating vascular calcification in diabetes [145]. O-GlcNAcylation plays crucial roles in the pathogenesis of human diseases, especially chronic diseases, including diabetes and cardiovascular diseases [146,147]. Elevation of O-GlcNAcylation is found in human diabetic carotid plaques [148] and diabetic mouse vasculature [149]. Coincidently, increased calcified plaques have been identified in diabetic patients [150] and diabetic mouse models [151]. Elevated glucose and insulin resistance, two hallmarks of diabetes, have been associated with increased intracellular stress and O-GlcNAcylation [152]. Using low dose-streptozotocin-induced mouse model of diabetes, we have demonstrated a novel causative link between chronic increases in vascular O-GlcNAcylation and vascular calcification in diabetes mellitus [145]. This finding adds to our current growing knowledge on how oxidative stress may regulate vascular calcification in different disease models.

Recent studies by our group and others have elucidated some of the mechanisms linking oxidative stress and vascular calcification. Demer and colleagues have shown that xanthine/xanthine oxidase induces osteogenic differentiation of bovine calcifying vascular cells via H_2O_2-activated signals [128]. Using primary mouse VSMC, we have demonstrated that H_2O_2 directly induces VSMC calcification, which is associated with the inhibition of VSMC-specific markers and the upregulation of bone-specific markers, indicating a phenotypic change of VSMC phenotype to an osteogenic phenotype [9]. Of note, oxidative stress modulates osteogenic differentiation of vascular cells and bone cells in an opposite manner [128,153], increasing VSMC calcification but inhibiting osteoblast differentiation. In VSMC, increased oxidative stress converted LDL into OxLDL, which promoted VSMC calcification [154]. In contrast, minimally oxidized low-density lipoprotein (mmLDL) and H_2O_2 increased intracellular oxidative stress and inhibited osteogenic differentiation of pre-osteoblasts or bone marrow stromal cells [128]. Consistently, we demonstrated that oxidative stress induced VSMC calcification by up-regulation of Runx2, which is mediated by the activation of the AKT/FOXO1/3 signaling axis via inhibiting Runx2 ubiquitination that leads to

increased Runx2 transactivity [9,155]. Interestingly, the in vitro oxidative stress in VSMC activates AKT by phosphorylation, normally a survival signal. The inhibition of AKT activation blocks Runx2 expression and osteogenic differentiation of VSMC [9]. These observations support the notion that activation of oxidative stress signaling while beneficial for VSMC within a short time frame, can be detrimental when chronically activated.

We have further demonstrated that oxidative stress-induced Runx2 promotes the expression of RANKL in calcifying VSMC, via direct binding to the RANKL promoter that enhances RANKL transcription [123]. The RANKL/RANK system is critical for the formation of osteoclasts, the bone-resorbing cells. Enriched OxLDL in atherosclerotic plaques was found to increase oxidative stress in VSMC and lead to increased RANKL in the atherosclerotic area [156]. Consistently, we have shown that Runx2 upregulates RANKL, which can attract osteoclast-like cells into the calcified area [123]. In atherosclerosis-prone ApoE$^{-/-}$ mice, increases in ROS were associated with up-regulation of Runx2 and RANKL in the calcified atherosclerotic lesions, accompanying the presence of tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells in this area [122,123]. On the contrary, smooth muscle-specific Runx2 deficiency down-regulated the expression of RANKL and was accompanied by decreased macrophage infiltration and reduced formation of osteoclast-like cells in the calcified lesions [122]. TRAP-positive cells have been previously observed in atherosclerotic lesions [157,158], however, their origin and functions are unknown. Our studies provided the evidence that VSMC-derived RANKL promoted macrophage migration and differentiation into osteoclasts [122,123], suggesting potential roles of RANKL-induced macrophage-derived osteoclasts in the atherosclerotic lesions [159]. Accordingly, oxidative stress plays a critical role in regulating VSMC function as well as VSMC crosstalk with macrophages and others within the cellular milieu that promote pathogenesis of vascular diseases.

3. Conclusion remarks

As a signaling second messenger as well as a durable and robust oxidizing agent, hydrogen peroxide exerts a wide array of effects on the physiology and pathology of the cardiovascular system, particularly in VSMC (Fig. 1). Nonetheless, therapeutic strategies are not established for the use of antioxidant regimen in clinical practice. As ROS, especially H_2O_2, at low physiological concentrations play an important role in intracellular signaling pathways for maintaining homeostasis of vascular cells, the use of antioxidants may not be as effective as expected. Future studies to comprehensively understand vascular redox biology, especially different spatiotemporal regulation of H_2O_2 production in VSMC under physiological and pathological conditions, should elucidate precise mechanisms by which this small redox molecule regulates VSMC inflammatory responses in the pathogenesis of cardiovascular diseases. In addition, unveiling H_2O_2–regulated signaling molecule profiles in VSMC and other vascular cells may provide novel molecular insights into the signaling cascades that mediate the pathogenic effects of H_2O_2, and thus leading to identification of molecular targets. Further investigation of the regulatory roles of VSMC in response to ROS including hydrogen peroxide involved in the interplay among other cell types are clearly required as well to develop therapeutic intervention targeted to cardiovascular pathologies.

Acknowledgments

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