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Review

Redox signaling in hypertension

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Abstract

Diseases such as hypertension, atherosclerosis and diabetes are associated with vascular functional and structural changes including endothelial dysfunction, altered contractility and vascular remodeling. Cellular events underlying these processes involve changes in vascular smooth muscle cell (VSMC) growth, apoptosis/anoikis, cell migration, inflammation, and fibrosis. Many stimuli influence cellular changes, including mechanical forces, such as shear stress, and vasoactive agents, of which angiotensin II (Ang II) appears to be amongst the most important. Ang II mediates many of its pleiotropic vascular effects through NAD(P)H oxidase-derived reactive oxygen species (ROS). Mechanical forces, comprising both unidirectional laminar and oscillatory shear, are increasingly being recognized as important inducers of vascular NO and ROS generation. In general, laminar flow is associated with upregulation of eNOS and NO production and increased expression of antioxidants glutathione peroxidase and superoxide dismutase, thereby promoting a healthy vascular wall and protecting against oxidative vascular injury. On the other hand, oscillatory shear is linked to increased ROS production with consequent oxidative damage, as occurs in hypertension. ROS function as important intracellular and intercellular second messengers to modulate many downstream signaling molecules, such as protein tyrosine phosphatases, protein tyrosine kinases, transcription factors, mitogen-activated protein kinases, and ion channels. Induction of these signaling cascades leads to VSMC growth and migration, expression of pro-inflammatory mediators, and modification of extracellular matrix. In addition, ROS increase intracellular free Ca^{2+} concentration, a major determinant of vascular reactivity. ROS influence signaling molecules by altering the intracellular redox state and by oxidative modification of proteins. In physiological conditions, low concentrations of intracellular ROS play an important role in normal redox signaling involved in maintaining vascular function and integrity. Under pathological conditions ROS contribute to vascular dysfunction and remodeling through oxidative damage. The present review describes some of the redox-sensitive signaling pathways that are involved in the functional and structural vascular changes associated with hypertension.

Keywords: Superoxide; Signal transduction; Angiotensin II; Kinases; Vascular smooth muscle cells; Blood pressure; Vascular remodeling; Inflammation

1. Introduction

One of the key characteristics of hypertension is increased peripheral resistance, due largely to a reduced lumen diameter of resistance vessels [1]. Since resistance is inversely proportional to the fourth power of the radius, a small change in diameter can significantly impact on vascular resistance. The small arteries and arterioles that determine peripheral resistance undergo both structural and functional changes in hypertension [2]. Examples of these changes include increased reactivity to contractile agents, impaired endothelial function, vascular smooth muscle growth, extracellular matrix deposition and vascular inflammation [3].

Over the past decade, the role of reactive oxygen species (ROS) in the cardiovascular system has been the subject of much research interest. The ROS 'family' encompasses various molecules, which have wide-ranging and divergent effects on cellular function. Within the cardiovascular system, the major effects of ROS include regulation of cell

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growth and differentiation, modulation of extracellular matrix production and breakdown, inactivation of nitric oxide (NO) and stimulation of many kinases [4]. Importantly, many of these effects are associated with pathological changes observed in hypertension.

The term 'oxidative stress' describes conditions involving chronically elevated ROS levels and is associated with cardiovascular disease. Patients with hypertension demonstrate increased levels of oxidative stress byproducts together with decreased activity of endogenous antioxidant enzymes in blood and mononuclear cells [5]. These patients also have indications of increased oxidative DNA damage when compared to normotensive individuals [5]. Direct measurements of ROS production from stimulated mononuclear cells showed that cells isolated from hypertensive patients had higher levels of $O_2^{\bullet-}$ production following stimulation with phorbol myristate acetate, angiotensin II (Ang II) or endothelin-1 when compared to normotensive subjects [6]. Similarly, patients with renovascular hypertension (who have elevated plasma renin activity and Ang II levels) demonstrate increased oxidative stress together with impaired endothelium-dependent vasodilatation [7]. Vascular ROS production is also elevated in a range of different experimental models of hypertension, including Ang II-induced [8,9], mineralocorticoid [10] and renovascular hypertension [11,12]. Thus, there is compelling evidence to suggest a role for ROS in the pathogenesis of hypertension.

Although all ROS are derived from the reduction of molecular oxygen, the different chemical properties of individual ROS have important implications for their role in cellular signaling. Both $O_2^{\bullet-}$ and $OH^{\bullet}$ have relatively short biological half-lives—the $OH^{\bullet}$ radical is particularly reactive, and thus unlikely to mediate effects distant from where it is produced. The charge on the superoxide anion makes it unable to cross cellular membranes except possibly through ion channels. In contrast, H_2O_2 has a longer biological life span than $O_2^{\bullet-}$ and $OH^{\bullet}$ and is able to diffuse across lipid bilayers. These distinct properties mean that different species of ROS are capable of activating different signaling pathways, which may then lead to divergent (and potentially opposing) consequences. For example, increased $O_2^{\bullet-}$ levels have long been known to inactivate the vasodilator, leading to endothelial dysfunction and vasoconstriction characteristic of many vascular diseases, including hypertension [13]. H_2O_2, however, has been shown to act as a vasodilator in a number of vascular beds, including cerebral, coronary and mesenteric arteries [14–16]. Thus, broadly attributing effects to 'oxidative stress' without examining the individual ROS-modulated signaling pathways involved may be a simplistic representation of what is actually occurring in vivo. The present review describes some of the redox-sensitive signaling pathways that are involved in the functional and structural vascular changes associated with hypertension.

2. Production and metabolism of ROS

ROS are produced by all vascular cell types, including endothelial, smooth muscle and adventitial cells, and can be formed by numerous enzymes. The most relevant sources of ROS with respect to vascular disease and hypertension appear to be xanthine oxidase, uncoupled endothelial NO synthase and NAD(P)H oxidase.

Xanthine oxidase is a metalloenzyme that catalyses the oxidation of hypoxanthine and xanthine to form $O_2^{\bullet-}$, and is known to be present in the vascular endothelium. Although xanthine oxidase-derived $O_2^{\bullet-}$ has been primarily studied in the context of ischemia–reperfusion injury and heart failure, there is also some evidence to suggest involvement in the endothelial dysfunction seen in hypertension. Spontaneously hypertensive rats (SHR) demonstrate elevated levels of xanthine oxidase activity in the mesenteric microcirculation, and this is associated with increased arteriolar tone [17]. Endothelial dysfunction in transgenic rats with overexpression of renin and angiotensinogen has also been associated with increased xanthine oxidase activity [18]. In addition to effects on the vasculature, xanthine oxidase may play a role in end-organ damage in hypertension. Both SHR and Dahl salt-sensitive rats exhibit increased xanthine oxidase activity in the kidney. In the SHR, long-term inhibition of xanthine oxidase with allopurinol reduced renal xanthine oxidase activity without lowering blood pressure, indicating that the increased renal ROS production was a consequence of hypertension rather than a contributing factor [19]. The finding that allopurinol can improve cardiac and renal hypertrophy in SHR whilst having a minimal impact on blood pressure [20] supports a role for xanthine oxidase in hypertensive end-organ damage rather than in the development of hypertension per se.

Nitric oxide synthase (NOS) can also contribute to ROS production, as all three NOS isoforms have been shown to be susceptible to the 'uncoupling' that leads to the formation of $O_2^{\bullet-}$ (rather than NO) under certain conditions [21]. For endothelial NOS, this process can be triggered in vitro through the absence of the co-factors L-arginine and tetrahydrobiopterin [22]. Importantly, uncoupling of endothelial NOS has been demonstrated in mice with DOCA-salt-induced hypertension [23]. The critical step in this uncoupling seems to be oxidation of tetrahydrobiopterin by $ONOO^-$, reducing the bioavailability of this critical cofactor [23,24]. Treatment with tetrahydrobiopterin improves blood pressure in both DOCA-salt hypertension and SHR [23,25].

Over the last decade, many studies have shown that the major source of ROS in the vascular wall is nonphagocytic NAD(P)H oxidase, which utilises NADH/NADPH as the electron donor to reduce molecular oxygen and produce $O_2^{\bullet-}$. Activation of this enzyme requires the assembly of both cytosolic (p47phox, p67phox or homologues) and membrane bound (gp91phox/Nox1/Nox4 and p22phox) subunits to form a functional enzyme complex. In the vasculature the NAD(P)H oxidase complex is at least partly

pre-assembled, as a significant proportion of NAD(P)H oxidase subunits are colocalized intracellularly in endothelial cells [26,27]. Activation of NAD(P)H oxidase is regulated by many vasoactive hormones, growth factors (platelet-derived growth factor, transforming growth factor-β) and mechanical stimuli (shear stress and stretch) [28]. The best studied pathway in vascular cells is that of NAD(P)H oxidase activation by Ang II, which has been shown to involve protein kinase C, phospholipase D, c-Src and receptor tyrosine kinases [29].

3. NAD(P)H oxidase and hypertension

There is a large body of evidence to support a role for ROS production, particularly from NAD(P)H oxidase, in the pathogenesis of hypertension. In rats made hypertensive by Ang II infusion, both NAD(P)H oxidase subunit expression and activity are increased [9,30], whilst administration of a NAD(P)H oxidase inhibitor reduces vascular $O_2^{\bullet-}$ production and attenuates Ang II-induced increases in blood pressure [31]. A number of recent studies used gene targeting approaches to confirm involvement of NAD(P)H oxidase isoforms in hypertension. In mice lacking the cytosolic subunit p47phox the hypertensive response to Ang II is markedly blunted, and these animals do not show the same increases in $O_2^{\bullet-}$ production and endothelial dysfunction observed in Ang II-infused wild-type mice [32,33]. Two recent complementary studies have provided evidence that a Nox1-containing isoform of NAD(P)H oxidase is involved in mediating the hypertensive response to Ang II. Nox1-deficient mice have reduced vascular $O_2^{\bullet-}$ production and blunted pressor responses to Ang II [34], whilst transgenic mice overexpressing Nox1 in smooth muscle show enhanced $O_2^{\bullet-}$ levels and blood pressure in response to Ang II [35]. Interestingly, while both studies supported a role for Nox1 in Ang II-mediated increases in blood pressure, their findings regarding the vascular hypertrophic response to Ang II differed. The hypertrophic response of the aorta to Ang II was increased in the Nox1-overexpressing mice [35], whereas Ang II-induced aortic hypertrophy was evident in both wild-type and Nox1-deficient animals [34]. A similar separation between the hypertrophic and hypertensive response was seen in mice with smooth muscle-specific overexpression of catalase, which demonstrated that whilst production of H_2O_2 was essential for Ang II-mediated vascular hypertrophy in vivo, it had no significant effect on blood pressure [36]. Thus, it appears that ROS may be involved in some of the pathways responsible for end-organ damage, independently of direct effects on the blood pressure.

Although the majority of studies investigating ROS generation in hypertension utilised pharmacological doses of exogenous Ang II, some studies implicated a role for the endogenous renin–angiotensin system in increasing ROS production during hypertension. In the 2-kidney 1-clip model of renovascular hypertension (a model which is associated with increased activation of the renin–angiotensin system), increased $O_2^{\bullet-}$ production from a gp91phox-containing NAD(P)H oxidase is associated with endothelial dysfunction and partly contributes to the elevation in blood pressure [12]. Similarly, in Dahl rats the development of

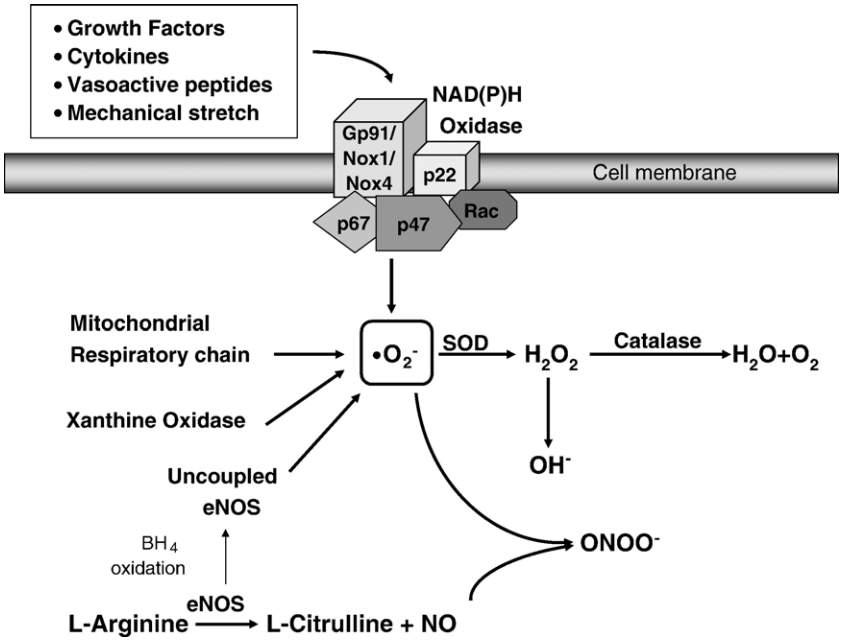

Fig. 1. Generation of $O_2^{\bullet-}$ and H_2O_2 from O_2 in vascular cells. Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase and uncoupled nitric oxide synthase (NOS) among others, have the potential to generate reactive oxygen species. NAD(P)H oxidase is a multisubunit enzyme, comprising gp91phox (or its homologues, Nox1 and Nox4), p22phox, p47phox, p67phox and p40phox, that is regulated by many stimuli, including vasoactive agents, such as Ang II. SOD, superoxide dismutase; BH_4, tetrahydrobiopterin.

salt-sensitive hypertension appears to be accompanied by activation of the local renin–angiotensin system, as treatment of these animals with an angiotensin receptor blocker reduced the salt-induced increases in aortic $O_2^{\bullet-}$ [37]. Treatment with an inhibitor of gp91phox-containing NAD(P)H oxidases also prevented the increased aortic $O_2^{\bullet-}$ production and expression of pro-inflammatory molecules seen in salt-sensitive hypertension [38]. Importantly, however, in the Dahl salt-sensitive hypertensive rats, neither the angiotensin receptor blocker candesartan nor the gp91phox inhibitor lowered the systolic blood pressure [37,38].

There is also evidence for ROS involvement in the pathogenesis of hypertension independent of Ang II actions. Spontaneously hypertensive rats (SHR) show increased $O_2^{\bullet-}$ production in both aortae and cerebral arteries when compared to normotensive Wistar–Kyoto controls [14,39]. Similarly, rats with DOCA salt-induced mineralocorticoid hypertension also show elevated vascular $O_2^{\bullet-}$ production that appears to be due to elevated NAD(P)H oxidase activity and may also involve subsequent uncoupling of endothelial NOS [23,40,41]. Other studies have identified endothelin-1 (acting at the ET_A receptor) as one of the key mediators of increased $O_2^{\bullet-}$ production in DOCA salt-induced hypertension [42,43]. Interestingly, these studies also indicate that endothelin-1-induced increases in ROS production can have effects on vascular structure and function independent of changes in blood pressure, as seen in some of the Ang II studies discussed above. Direct infusion of endothelin-1 can increase NAD(P)H oxidase-dependent $O_2^{\bullet-}$ production; however, preventing this increase in ROS generation does not stop the development of hypertension in these animals [44]. Overexpression of human endothelin-1 in mice also induces vascular remodeling and impairs endothelial function (via the activation of NAD(P)H oxidase), an effect that occurs independently of significant increases in blood pressure [45] (Fig. 1).

Together, these studies underlie the complexity of interactions between the renin–angiotensin system, ROS, and other factors in hypertension. Furthermore, they provide additional support for drawing a distinction between the different signaling pathways involved in the acute hemodynamic response to pro-hypertensive stimuli, and those responsible for mediating changes in vascular architecture and end-organ damage.

4. ROS signaling in the CNS

The effects of ROS signaling on blood pressure are not limited to vascular cells. It is well-established that Ang II can control both cardiovascular and volume homeostasis through acting on the central nervous system, although the signaling pathways involved in these effects are relatively poorly characterised. The first demonstration that Ang II could increase ROS production in the brain used intracerebroventricular administration of adenoviral vectors encoding SOD isoforms to demonstrate that $O_2^{\bullet-}$ was responsible for mediating the pressor effects of centrally administered Ang II [46]. The same authors later demonstrated that both the increased $O_2^{\bullet-}$ production and the subsequent cardiovascular responses were reliant on a Rac1-dependent NAD(P)H oxidase [47]. In addition to exerting an acute effect when administered directly into the brain, a chronic subpressor dose of Ang II can also increase central $O_2^{\bullet-}$ production and lead to the development of hypertension [48]. The involvement of ROS signaling in the central nervous system during hypertension does not seem to be limited to situations involving elevated Ang II. The rostral ventrolateral medulla (RVLM) (an area of the brain stem involved in controlling basal sympathetic vasomotor activity) shows elevated levels of ROS in stroke-prone SHR compared to WKY, and scavenging of $O_2^{\bullet-}$ in the RVLM reduces both sympathetic nerve activity and blood pressure [49].

5. Hemodynamic influences on redox signaling

Another intriguing aspect of redox signaling is the role of biomechanical forces. Blood vessels are continually exposed to mechanical stresses, and alterations in these forces are thought to be important in vascular remodeling in both physiological conditions, such as exercise training, and in pathological conditions, such as hypertension, atherosclerosis and diabetes [50,51] . The two main forces acting on the blood vessel wall are shear stress (generated by movement of blood through the vessel lumen) and stretch (determined by luminal pressure). In injured vessels, vascular stretch affects both the endothelium and vascular smooth muscle, whilst in undamaged vessels shear stress is thought to act primarily at the endothelial layer. Shear stress and cyclic mechanical stretch influence vascular function and structure, in part, by stimulating production of NO and ROS [52].

5.1. Mechanical forces and nitric oxide

Laminar shear induces an increase in NO production through increased activation and expression of eNOS. Acutely, laminar shear activates eNOS through Ca^{2+}-dependent and Ca^{2+}-independent mechanisms [53,54]. Over the long term, shear upregulates eNOS/NO production, by stimulating a transient increase in eNOS mRNA transcription and a sustained increase in eNOS mRNA stability. Oscillatory shear also stimulates an acute increase in NO production and upregulation of eNOS. However, the signaling processes underlying these effects are different [55]. Whereas laminar shear involves activation of c-Src-MAP kinase pathways, oscillatory shear increases endothelial production of $^{\bullet}O_2^-$ and H_2O_2, which stimulates eNOS expression.

Exercise increases vascular shear stress and is an important physiological mechanical activator of endothelial NO production and inducer of eNOS expression. These

processes contribute to vascular changes associated with exercise training, including physiological remodeling, vasodilation, improved organ blood flow, angiogenesis, arteriogenesis and vascular protection [51]. In contrast disturbed flow profiles, such as in hypertension and atherosclerosis, are associated with opposite effects, where oscillatory shear promotes oxidative stress and oxidative vascular damage.

5.2. Mechanical forces, superoxide, hydrogen superoxide and peroxynitrite

In addition to NO generation, mechanical forces stimulate production of $^{\bullet}O_2^-$ and H_2O_2, in intact vessels exposed to elevated intraluminal flow [56] and in cultured endothelial and VSMCs exposed to shear stress [57]. However, the source of ROS remains controversial and may derive from NAD(P)H oxidase, xanthine oxidase, mitochondrial enzymes or from other systems, such as uncoupled NOS [57,58]. Oscillatory shear stress of human umbilical endothelial vein endothelial cells over 24 h induced a progressive increase in NADPH oxidase activity [57]. Oscillatory shear stress increases expression of p22phox, gp91phox and Nox4 in endothelial cells, whilst pulsatile shear stress downregulates these subunits [59,60]. Oscillatory shear stress has been implicated in vascular inflammation through the activation of a Nox1-containing oxidase [61].

Superoxide rapidly reacts with NO to form the highly reactive intermediate peroxynitrite ($ONOO^-$), which has recently been shown to be an important signaling molecule in shear/flow-dependent activation of MAP kinases (JNK) [62], MMPs [63] and adhesion molecules [64]. Downstream signalling events depend on the concentration of ONOO- formed. Laminar flow is associated with low concentrations of $ONOO^-$, which play a protective role by inhibiting activation of adhesion molecules. On the other hand, oscillatory shear stress is associated with sustained $^{\bullet}O_2^-$ production, which in the presence of NO, enhances peroxynitrite formation and protein nitration [65]. These processes may contribute to vascular damage and atherosclerotic lesion formation.

Whereas oscillatory shear promotes oxidative stress, $ONOO^-$ formation and oxidative vascular damage, laminar shear seems to have a predominant antioxidant effect, at least in the long term. Laminar shear stimulates expression of the cytosolic copper/zinc-containing superoxide dismutase (SOD) and extracellular SOD, major sources of cytoplasmic and extracellular $^{\bullet}O_2^-$ scavenging, respectively [66]. Laminar flow also increases expression and intracellular levels of glutathione (GSH) peroxidase, responsible for H_2O_2 scavenging [52,67]. Together with NO-stimulating effects, this may be another mechanism whereby laminar flow protects against vascular injury.

Shear stress-induced ROS production has numerous functional actions in the vasculature. Acutely, the generation of H_2O_2 has been implicated as a mediator of flow-induced vasodilatation in coronary and cerebral vessels [68,69,114]. Redox signaling is also involved in more chronic effects of altered flow, with a recent study demonstrating that flow-induced vascular remodeling involves the production of ROS from a p47phox-containing NAD(P)H oxidase and the subsequent activation of matrix metalloproteinases [63].

Vascular stretch has also been shown to activate redox-sensitive signaling pathways. In endothelium-denuded strips of vascular smooth muscle isolated from bovine coronary arteries, passive stretch induces contraction via the activation of NAD(P)H oxidase and ERK1/2 [70]. Similarly, Ungvari et al. demonstrated that high intraluminal pressures also cause $O_2^{\bullet-}$ production via activation of NAD(P)H oxidase in intact isolated vessels, an effect that is independent of the local renin–angiotensin system [71]. Activation of mechanically sensitive redox signaling pathways may thus contribute to some of the maladaptive responses to altered hemodynamics in hypertension.

5.3. Mechanotransduction, nitric oxide, reactive oxygen species and hypertension

Increased vascular pressure in hypertension is associated with stretch of endothelial and VSMCs, which can directly activate NAD(P)H oxidase to generate ROS. This effect may be amplified by activation of the renin–angiotensin system. Increased oxidative stress in reponse to stretch contributes to activation of pro-inflammatory transcription factors, activation of growth-promoting MAP kinases, upregulation of pro-fibrogenic mediators and altered vascular tone, important processes contributing to the vascular phenotype associated with hypertension.

6. Molecular targets of ROS

6.1. Mitogen-activated protein kinases

Mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases associated with many signaling cascades controlling cell proliferation, differentiation and death. Activation of MAP kinases is dependent on a series of upstream phosphorylation events, allowing both the interaction of multiple signaling pathways and the potential for signal amplification [72]. Importantly, all of the major MAP kinases in the vasculature, extracellular-signal regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinase (JNK), p38MAP kinase and ERK5, are activated by growth factors such as Ang II and platelet-derived growth factor (PDGF) [73–75].

Although the ability of exogenously generated ROS to activate MAP kinases has been known for over a decade [76], fewer studies have investigated MAP kinases as targets of intracellular ROS. In VSMCs, Ang II-induced p38MAP kinase activation is dependent on H_2O_2 [73], most likely

derived from NAD(P)H oxidase [77]. Activation of this pathway may be affecting the vasculature at multiple levels. The constrictor effect of Ang II is mediated by both the activation of p38MAP kinase [78] and production of H_2O_2 [79] and, thus, may contribute to enhanced reactivity seen in hypertension. p38MAP kinase is also an important regulator of collagen synthesis in SHR [80], and MAP kinase activation by ROS is crucial for mediating VSMC growth. Thus, activation of redox-sensitive p38MAP kinase could be involved in both functional and structural changes in hypertension.

A few studies also investigated the signaling pathways that lie upstream of p38MAP kinase activation—in particular, identifying the source of the ROS responsible for activating the kinase. Early studies had shown an attenuation of p38MAP kinase phosphorylation by the NAD(P)H oxidase inhibitor diphenylene iodonium, and this was later confirmed by other studies utilizing a molecular biology approach. Ang II-stimulated ROS production and p38MAP kinase activation were prevented by antisense to either p22phox or Nox1, membrane-bound subunits of NAD(P)H oxidase [77,81]. The cytosolic NAD(P)H oxidase subunit p47phox is also implicated in the activation of the MAP kinase signaling cascade, as interaction between p47phox and actin was required for Ang II-mediated assembly of NAD(P)H oxidase and the subsequent ROS production and phosphorylation of p38MAP kinase [60].

In contrast, the potential for activation of ERK1/2 by ROS is more poorly characterised. Some studies have shown that ERK1/2 activation by Ang II is redox-independent [73,77], whereas ERK1/2 activation by pulsatile stretch involves ROS [82].

6.2. Protein tyrosine kinases

In addition to MAPKs, there are many other kinases that are regulated by ROS in the vasculature, including both receptor and non-receptor tyrosine kinases. Receptor tyrosine kinases comprise the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor-β (PDGFR-β). In classical growth factor-mediated responses, there is evidence that the production of H_2O_2 is required for ligand-stimulated signal transduction [75]. However, ligand-independent signaling can also activate these receptor tyrosine kinases. By binding to the G protein-coupled AT_1 receptor, Ang II activates receptor tyrosine kinases through transactivation of growth factor receptors such as EGFR and PDGFR-β [83]. This transactivation allows the EGFR to act as a scaffold for other signaling proteins, ultimately leading to activation of MAP kinases in VSMCs [83]. There is now a body of evidence indicating that this transactivation of receptor tyrosine kinases may be mediated by ROS.

Exogenously generated ROS can cause EGFR tyrosine phosphorylation, and Ang II-induced EGFR phosphorylation is sensitive to inhibition by antioxidants [84]. This process was shown to involve another redox-sensitive enzyme, the non-receptor tyrosine kinase Src. Activation of EGFR by ROS can lead to activation of various MAP kinase pathways [84,85].

A mechanism for EGFR transactivation involving release of heparin-binding EGF (HB-EGF) has been proposed [86]. HB-EGF is a potent mitogen for VSMCs that is formed as a transmembrane precursor and requires proteolytic cleavage to produce the active growth factor. This proteolytic cleavage is catalysed by metalloproteinases, and Ang II-stimulated activation of ERK1/2 and p38MAP kinase via EGFR transactivation is dependent on metalloproteinase activity and HB-EGF release [74]. This pathway appears to involve ROS, as either a metalloproteinase inhibitor or a HB-EGF-neutralising antibody can block activation of the EGFR by H_2O_2 [87]. More recently, a study from the same group demonstrated that elevations in intracellular Ca^{2+} and ROS production were essential for metalloproteinase-dependent shedding and EGFR transactivation by Ang II [88].

Non-receptor tyrosine kinases can also be regulated by ROS. Several studies have implicated Src tyrosine kinases as contributing to H_2O_2- and Ang II-induced EGFR transactivation [85]. c-Src also appears to be involved in the signaling pathway downstream of EGFR transactivation by Ang II, as EGFR inhibition attenuates the Ang II-induced phosphorylation of both c-Src and ERK1/2 [89]. This dual role of the redox-sensitive kinase Src (where it is seen both upstream and downstream of EGFR transactivation) is also seen in the signaling cascade responsible for the activation of NAD(P)H oxidase by Ang II in VSMCs29. c-Src regulates Ang II-induced NAD(P)H oxidase activity by stimulating p47phox phosphorylation and translocation [90]. c-Src activation may be involved in the stimulation of a number of redox-sensitive signaling cascades in hypertension. Indeed, both c-Src phosphorylation and Src-dependent ERK1/2 activation by Ang II are elevated in hypertension [89]. c-Src may also be involved in mechanically induced signaling pathways. Cyclic strain of endothelial cells induces phosphorylation of c-Src and the related kinase Pyk2 in a ROS-dependent manner [91]. Increased vascular strain can also increase Src-dependent activation of ERK1/2 [92] and lead to expression of the early-response gene c-fos [93]. Activation of redox-sensitive tyrosine kinases may thus be playing a complicated variety of roles in mediating some of the vascular changes seen in hypertension.

6.3. Protein tyrosine phosphatases

Currently the best established direct molecular targets of ROS are protein tyrosine phosphatases. Protein–tyrosine phosphorylation is a major mechanism for post-translational modification of proteins and plays a critical role in regulating cell proliferation, differentiation, migration, and transformation. The level of tyrosine phosphorylation in cells is controlled by the tightly regulated balance between

protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) [94]. By dephosphorylating PTK substrate proteins, PTPs counteract effects of PTK activity. Hence, PTPs may be considered as negative regulators and terminators of a signaling process initiated by PTK activation. Exposure of cells to low doses of oxidants or thiol-directed agents induces an increase in tyrosine phosphorylation due to PTP inactivation.

PTPs are a large, structurally diverse family of receptor and non-receptor enzymes that are critical regulators of multiple signaling pathways [94]. Because of their particular structure, PTPs are susceptible to oxidation and inactivation by ROS. All PTPs possess a conserved 230-amino acid domain that contains a reactive and redox-regulated cysteine, which catalyzes the hydrolysis of protein phosphotyrosine residues by the formation of a cysteinyl-phosphate intermediate [95]. This cysteine forms thiol phosphate, an intermediate in the dephosphorylation reaction of PTPs. Oxidation of this cysteine residue to sulfenic acid by H_2O_2 renders the PTP completely inactive [95] (Fig. 2). Since the oxidation of PTP is reversible, PTPs exist in two forms: an active state with a reduced cysteine or an inactive state with an oxidized cysteine. Activation and inactivation of PTPs are regulated by extracellular signals, including Ang II [96] and EGF and ROS play major roles as secondary messengers in this process [96]. Lee et al. [97] demonstrated that EGF-induced PTP1B inactivation is dependent on reversible oxidation of cysteine residues by H_2O_2. Recent studies suggest that PTP1B may be more efficiently regulated by $^{\bullet}O_2^-$ than by H_2O_2 [57]. Peroxynitrite rapidly and irreversibly inhibits PTPs, supporting the role of this ROS in oxidative damage.

Besides soluble phosphatases, receptor PTP (RPTP) are modulated by oxidative stress [94]. A model has been proposed in which oxidative stress induces a conformational change in RPTPa-D2, leading to stabilization of RPTPa dimers, and thus to inhibition of RPTPa activity [94]. In addition, inactivation of PTPs is involved in oxidative stress-induced activation of several PTK such as the EGFR, insulin receptor, Lck and Fyn. This is particularly important with respect to Ang II, which mediates many of its signaling events in vascular cells through EGFR transactivation. H_2O_2 has also been shown to regulate MAP kinases through inhibition of PTP activity of CD45, SHP-1 and HePTP [97]. Thus, activation of vascular MAP kinases by Ang II may be mediated, in part, through redox-dependent inactivation of PTPs.

6.4. Extracellular matrix and metalloproteinases

Modulation of the extracellular matrix is an important component of vascular remodeling seen in hypertension. Matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading extracellular matrix components. These enzymes are secreted as inactive pro-enzymes and require cleavage of the pro-domain for activation. MMP9 was recently suggested to play a critical role in the early stages of vascular remodeling due to increased pressure [98]. The activity of MMPs is tightly controlled, and

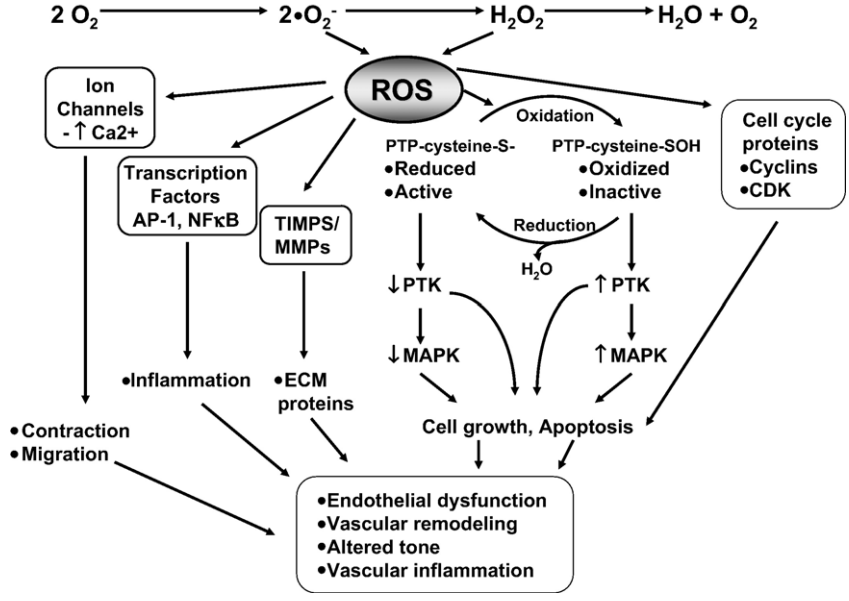

Fig. 2. Redox-dependent signaling pathways in vascular smooth muscle cells. Intracellular reactive oxygen species (ROS) modify the activity of protein tyrosine kinases (PTK), such as Src, Ras, JAK2, Pyk2, PI3K, and EGFR, as well as mitogen-activated protein kinases (MAPK), particularly p38MAPK, JNK and ERK5. These processes probably occur through oxidation/reduction of protein tyrosine phosphatases (PTP), which are susceptible to oxidation and inactivation by ROS. ROS also influence gene and protein expression by activating transcription factors, such as NF-κB, activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1). ROS stimulate ion channels, such as plasma membrane Ca^{2+} and K^+ channels, leading to changes in cation concentration. Activation of these redox-sensitive pathways results in numerous cellular responses which, if uncontrolled, could contribute to hypertensive vascular damage. ECM, extracellular matrix; MMPs, matrix metalloproteinases; TIMP, tissue inhibitor of matrix metalloproteinase.

regulated at multiple levels—transcription, protein synthesis, and formation of active zymogens. Exogenous ROS can activate MMP2 and MMP9 secreted from cultured VSMCs [99]. Endogenously produced ROS also have an effect on MMPs. Cyclic stretch of VSMCs increases the transcription and release of MMP2, an effect that is absent in cells from mice lacking the NAD(P)H oxidase component p47phox [100]. Similarly, activation of MMP2 by Ang II requires a p47phox-containing oxidase [101]. Together, these studies indicate that redox-sensitive signaling pathways are involved in the modulation of the extracellular matrix.

In addition to causing activation of the MAP kinase pathway and vascular hypertrophy, EGFR transactivation may also be involved in some of the functional alterations seen in hypertension. In isolated resistance arteries, increases in pressure caused MMP-mediated HB-EGF release, transactivation of the EGFR and the development of myogenic tone [102]. Myogenic tone is the contraction of resistance arteries to increased pressure and is a critical aspect of the control of peripheral resistance [103]. Thus, the ability of MMPs to be activated by ROS may impact upon both structural and functional changes to the vasculature in hypertension.

6.5. Inflammatory gene expression

It is becoming clear that vascular inflammation plays an important role in triggering fibrosis and remodeling during hypertension. Expression of adhesion molecules and recruitment of inflammatory cells are just two of the many cellular processes seen in hypertension-induced vascular inflammation. One of the major factors underlying this vascular inflammation is modulation of pro-inflammatory gene expression via redox-sensitive transcription factors.

As with many of the vascular changes seen during hypertension, Ang II plays an important role in modulating the expression of proinflammatory molecules. Treatment of human VSMCs with Ang II induced release of interleukin-6 (IL-6), a cytokine that causes the recruitment of inflammatory cells into the vessel media. The release of IL-6 required the production of ROS and activation of the redox-regulated transcription factor NF-κB [104]. Other signaling cascades involved in the transcription of proinflammatory genes include the janus kinase/signal transducers and activators of transcription factors (JAK/STAT) pathways, which are activated by exogenous H_2O_2 in cultured fibroblasts [105]. The JAK/STAT cascade is also activated by ROS produced by platelet-derived growth factor and Ang II [104,105]. Ang II-induced JAK/STAT activation was prevented by inhibiting a p47phox-containing NAD(P)H oxidase, an effect that also inhibited the synthesis and release of IL-6 by Ang II [104].

A number of the pathways responsible for adhesion molecule expression are also redox-sensitive. Cyclic strain of endothelial cells elevates expression of ICAM-1 expression in a ROS-dependent manner [106]. The increase in VCAM-1 expression by Ang II treatment of cultured fibroblasts is mediated by production of H_2O_2 and subsequent activation of NF-κB [107]. A similar effect is seen in vivo, with Ang II-infused hypertensive rats showing increased VCAM-1 expression due to NF-κB-mediated transcriptional events [108].

We now have evidence to suggest that vascular inflammation plays a critical role in Ang II-induced remodeling of resistance arteries. Mice deficient in macrophage colony-stimulating factor (a monocyte chemotactic factor) exhibit reduced inflammation. When infused with Ang II, these animals show attenuated vascular remodeling (both media/lumen ratio and medial thickness) and VCAM-1 expression in comparison to Ang II-infused wild-type controls [109]. Interestingly, although this study showed that suppression of the vascular inflammatory response could blunt Ang II-induced increases in blood pressure, other work has shown that the inflammatory responses to Ang II can occur independently of changes in blood pressure. Infusion of Ang II in the presence of the NAD(P)H oxidase inhibitor gp91ds-tat caused a smaller increase in aortic ICAM-1 expression and macrophage filtration than seen in animals treated with Ang II alone [110]. However, these improvements occurred without NAD(P)H oxidase inhibition having any effect of blood pressure, further supporting the concept that multiple redox-sensitive signaling pathways are activated in hypertension, and that these may have divergent effects on function and structure.

6.6. Cell cycle proteins

The regulation of VSMC growth includes both apoptosis and proliferative pathways, and the balance between each determines the magnitude of cell growth. Several studies have demonstrated that the cell cycle can be arrested in response to ROS [111]. Alteration in redox state also results in delayed progression through G1 to S phases as well as G2 arrest. These effects are mediated through inhibition of cyclin E/CDK2 and cyclin B/CDK1.

6.7. Ion channels

ROS also activate ion channels, a process that is critical for some of the vasoactive effects of ROS. In particular, H_2O_2 has been proposed as a potential endothelial-derived hyperpolarising factor due to its ability to activate potassium channels in the vasculature. Calcium-activated potassium channels can be activated by H_2O_2 in a number of vascular beds, including the cerebral [112], coronary [113] and mesenteric [16] vasculature. Importantly, endogenously generated H_2O_2 (e.g., in reponse to flow) is also capable of causing hyperpolarisation and subsequent changes in vascular tone [68]. This implies a physiological role for this

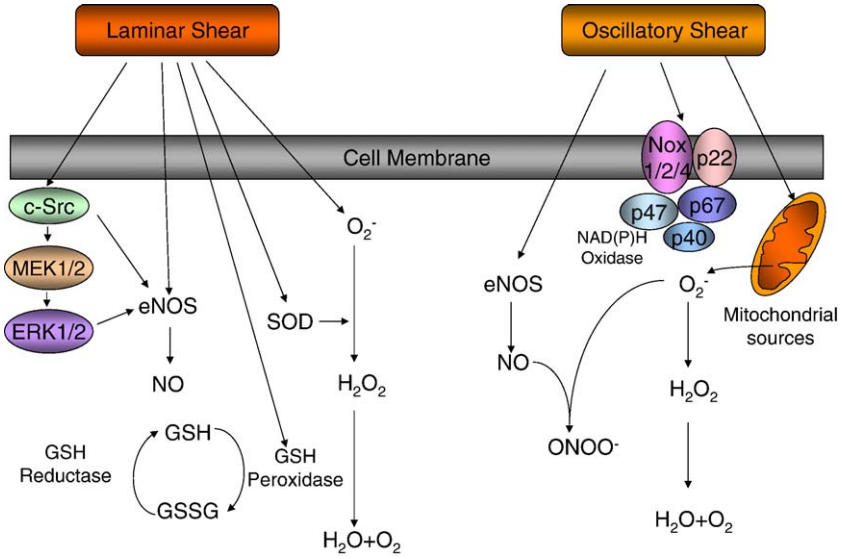

Fig. 3. Mechanisms whereby mechanical forces influence generation of nitric oxide (NO) and reactive oxygen species (ROS) in vascular cells. Unidirectional laminar shear stress increases NO production by stimulating activation of eNOS and by increasing eNOS mRNA and protein expression. These effects are mediated via c-Src-MAP kinase pathways. Laminar shear also increases expression/activity of SOD and glutathione peroxidase (GSH), thereby contributing to antioxidant effects. Oscillatory shear causes a sustained increase in ROS production, derived in part from NAD(P)H oxidase, xanthine oxidase, uncoupled eNOS and mitochondrial enzymes. Oscillatory shear-induced ROS formation interacts with NO to form peroxynitrite (ONOO−), which under physiological conditions is vascular protective, but under pathological conditions contributes to oxidative damage. GSH, glutathione; GSSG, GSH disulfide; SOD, superoxide dismutase.

mechanism, although how it is affected in the longer term by hypertension is currently unknown.

7. Conclusions

Reactive oxygen species, particularly $O_2^{\bullet -}$ and H_2O_2, function as second messengers activating numerous signaling molecules such as tyrosine kinases, tyrosine phosphatases, MAP kinases and ion channels, primarily through oxidative modification of proteins and activation of transcription factors. These signaling molecules play an important role in vascular (patho)biology. In hypertension, activation of pro-oxidant enzymes such as NAD(P)H oxidase, NOS, xanthine oxidase and mitochondrial enzymes or altered thioredoxin and glutathione systems results in increased ROS formation, which have damaging actions on the vasculature. Stimuli that activate pro-oxidant systems to generate ROS involve vasoactive agents, such as Ang II, and mechanical forces, such as shear stress. Laminar shear induces NO production, upregulation of anti-oxidant systems and vascular protective actions, whereas oscillatory shear promotes oxidative stress, ONOO-formation and oxidative damage. Hence, laminar flow-mediated redox signaling may be important physiologically, whereas oscillatory shear-induced redox signaling, together with Ang II activation of redox-sensitive molecules, may play a pathophysiological role in vascular injury and inflammation. Oxidative stress contributes to vascular damage by promoting cell growth, extracellular matrix protein deposition, activation of matrix metalloproteinases, inflammation, endothelial dysfunction, and increased vascular tone, characteristic features of the vascular phenotype in hypertension. Although inconclusive at present, treatment strategies to alter ROS bioavailability by decreasing production and/or by increasing radical scavenging may down-regulate signaling through ROS, thereby preventing further vascular injury and hypertension (Fig. 3).

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