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Forum Review

Reactive Oxygen Species as Mediators of Calcium Signaling by Angiotensin II: Implications in Vascular Physiology and Pathophysiology

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ABSTRACT

Reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hydroxyl radical, and reactive nitrogen species, such as nitric oxide and peroxynitrite, are biologically relevant O_2 derivatives increasingly being recognized as important in vascular biology through their oxidation/reduction (redox) potential. All vascular cell types produce ROS primarily via membrane-associated NAD(P)H oxidase. ROS influence vascular function by modulating contraction/dilation, cell growth, apoptosis/anoikis, migration, inflammation, and fibrosis. An imbalance in redox state where prooxidants overwhelm antioxidant capacity results in oxidative stress. Oxidative excess and associated oxidative damage are mediators of altered vascular tone and structural remodeling in many cardiovascular diseases. ROS elicit these effects by influencing intracellular signaling events. In addition to modulating protein tyrosine kinases, protein phosphatases, mitogen-activated protein kinases, and transcription factors, ROS are important regulators of intracellular Ca^{2+} homeostasis and RhoA/Rho kinase signaling. ROS increase vascular $[Ca^{2+}]_i$ by stimulating inositol trisphosphate-mediated Ca^{2+} mobilization, by increasing cytosolic Ca^{2+} accumulation through sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibition, and by stimulating Ca^{2+} influx through Ca^{2+} channels. Increased ROS generation enhances Ca^{2+} signaling and up-regulates RhoA/Rho kinase, thereby altering vascular contractility and tone. The present review discusses the importance of ROS in angiotensin II signaling in vascular biology and focuses specifically on the role of oxidative stress in Ca^{2+} signaling in the vasculature. *Antioxid. Redox Signal.* 7, 1302–1314.

INTRODUCTION

CARDIOVASCULAR DISEASES, such as hypertension, atherosclerosis, hyperlipidemia, postischemic reperfusion injury, and cardiac failure, are associated with vascular changes characterized by endothelial dysfunction, altered vascular tone, structural remodeling, mechanical alterations, and vascular inflammation (40, 85). Vascular smooth muscle cells (VSMCs), because of their dynamic, plastic, and multifunctional characteristics, are critically involved in these processes (11). Of the many humoral factors regulating VSMC function, angiotensin II (Ang II) is of major importance. Ang II, the final effector hormone of the renin–angiotensin system, was originally described as a potent vasoconstrictor. It is now well recognized that Ang II has pleiotropic actions in multiple organ systems. In the vasculature, Ang II induces contraction, cell growth, migration, and differentiation (68, 89). It is also proinflammatory and profibrotic, and it stimulates production of other growth factors (epidermal growth factor and platelet-derived growth factor) and vasoactive agents (endothelin-1) (25).

Under physiological conditions, Ang II regulates vascular tone and maintains structural integrity. In pathological condi-

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tions, Ang II plays a major role in endothelial dysfunction and vascular damage. At the subcellular level, these processes are mediated by complex networks of interacting signaling pathways. Among the numerous signaling molecules involved in Ang II-induced vascular actions, reactive oxygen species (ROS) appear to be critical (31). The significance of Ang II in vascular pathology associated with hypertension, atherosclerosis, and diabetes is supported by experimental and clinical studies demonstrating that angiotensin-converting enzyme inhibitors and Ang II type 1 (AT_1) receptor blockers not only improve clinical status in patients, but also regress arterial remodeling, improve endothelial function, reduce vasomotor tone, decrease inflammation, and normalize aberrant signaling events in VSMCs (16, 72, 88). Many of these effects have been attributed to inhibition of superoxide production and antioxidant actions.

Within the cardiovascular system, superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and the reactive nitrogen species, nitric oxide (NO) and peroxynitrite ($ONOO^-$), are biologically important (31, 101). Normally vascular ROS are produced in a controlled manner at low concentrations and function as signaling molecules regulating VSMC contraction–relaxation and VSMC growth. In pathological conditions, increased ROS production leads to endothelial dysfunction, increased contractility, VSMC growth, monocyte migration, lipid peroxidation, endothelial cell anoikis (shedding), inflammation, and increased deposition of extracellular matrix proteins, major processes contributing to vascular damage in cardiovascular disease (47, 90).

Ang II stimulates production of ROS in all vascular cell types, primarily through activation of cell membrane-associated NADPH oxidase (50). Endogenously produced ROS are now considered second messengers that influence numerous reduction–oxidation (redox)-sensitive molecules in Ang II-mediated signaling pathways. ROS targets in vascular cells include ion channels, RhoA, protein tyrosine kinases, mitogen-activated protein (MAP) kinases, protein phosphatases, and transcription factors (24, 69). These signaling molecules are involved in regulating vascular function. The present review discusses redox-sensitive events important in Ang II signaling in vascular physiology and pathophysiology. We will focus specifically on Ca^{2+} signaling and oxidant stress and will discuss the role of these processes in Ang II-regulated vascular tone in health and disease. Although the cardiac, renal, endocrine, and central nervous systems are also major Ang II-regulated targets for oxidative damage by ROS, these systems will not be discussed here, and the reader is referred to excellent reviews on these systems (31, 79, 101, 106).

ROS AND OXIDATIVE STRESS IN THE VASCULATURE

ROS are formed as intermediates in redox processes, leading from oxygen to water (26). The univalent reduction of oxygen, in the presence of a free electron (*e*), yields $\cdot O_2^-$, H_2O_2, and ·OH. $\cdot O_2^-$, which is highly unstable and short-lived, is water-soluble and acts either as an oxidizing agent, where it is reduced to H_2O_2, or as a reducing agent, where it donates its extra electron to form $ONOO^-$ with NO (18). H_2O_2 is produced mainly from dismutation of $\cdot O_2^-$. This reaction can be spontaneous or it can be catalyzed by superoxide dismutase (SOD), of which there are three isoforms: CuZnSOD, MnSOD, and extracellular SOD (EC-SOD) (15). The vascular media is rich in EC-SOD and probably contributes to vascular H_2O_2 formation (76). H_2O_2 is lipid-soluble, crosses cell membranes, and has a longer half-life than $\cdot O_2^-$. It is scavenged by catalase and by glutathione peroxidase and can also be reduced to generate the highly reactive ·OH in the presence of metal-containing molecules (26). ·OH is extremely reactive, and unlike $\cdot O_2^-$ and H_2O_2, which travel some distance from their site of generation, ·OH induces local damage where it is formed.

In the vasculature, production of $\cdot O_2^-$, H_2O_2, NO, $OONO^-$, and ·OH are tightly regulated by antioxidants such as SOD, catalase, thioredoxin, glutathione, antioxidant vitamins, and other small molecules (15, 70, 76). Under physiological conditions, the rate of ROS production is balanced by the rate of elimination. However, an imbalance between ROS formation and the ability to defend against them by antioxidants results in increased bioavailability of ROS, leading to a state of oxidative stress. The pathogenic outcome of oxidative stress is oxidative damage, a major cause of vascular injury in cardiovascular diseases.

VASCULAR PRODUCTION OF ROS BY Ang II

Vascular ROS are produced in endothelial and adventitial cells and VSMCs (41, 50) and derived predominantly from vascular NAD(P)H oxidase (50). NAD(P)H oxidase is a multisubunit enzyme (6, 30) that catalyzes the production of $\cdot O_2^-$ by the one-electron reduction of oxygen using NAD(P)H as the electron donor: $2O_2 + NAD(P)H \rightarrow 2O_2^- + H^+ + NAD(P)^+$. Vascular NAD(P)H oxidase comprises at least four components: cell membrane-associated p22phox (phox for phagocyte oxidase) and the electron transfer subunit gp91phox [or gp91phox (nox2) homologues, nox1, and nox4 (Nox for NAD(P)H oxidase)], and cytosolic subunits, p47phox and p67phox (6, 30). The distribution of these subunits varies between vascular cell types and between vascular beds (50). In endothelial and adventitial cells, p47phox, p67phox, p22phox, and gp91phox/nox1/nox4 are present (50, 52). The situation is more complex in VSMCs, where the major subunits are not always detected. Only p47phox and p22phox seem to be consistently expressed (50). In rat aortic VSMCs, p22phox and p47phox, nox1 and nox4, but not gp91phox (nox2), are present (38, 49), whereas in human resistance arteries, all of the major subunits, including gp91phox, are expressed (91, 93). Although initial studies suggested that nox1 is a subunit-independent low-capacity $\cdot O_2^-$-generating enzyme involved in the regulation of mitogenesis (78), recent data indicate that nox1 requires p47phox and p67phox and that it is regulated by NoxO1 (Nox organizer 1) and NoxA1 (Nox activator 1) (8). The exact role of NoxO1 and NoxA1 in vascular cells is currently unknown. Nox1 may be important in pathological processes as it is sig-

nificantly up-regulated in vascular injury (75, 81). Nox4 appears to be abundantly expressed in all vascular cell types (38, 75) and may be important in constitutive production of $\cdot O_2^-$ in nonproliferating cells. Ago *et al.* recently reported that Nox4 is the major catalytic component of endothelial NAD(P)H oxidase (1). A unique p67phox homologue has also been identified, but it is not yet known whether this isoform is present in vascular cells (29). The functional significance of NAD(P)H oxidase subunit homolgues in the vasculature is presently unclear and awaits further clarification. Unlike phagocytic NAD(P)H oxidase, which is activated only upon stimulation and which generates $\cdot O_2^-$ in a burst-like manner extracellularly (50), vascular oxidases are constitutively active and preassembled, produce $\cdot O_2^-$ intracellularly in a slow and sustained fashion, and act as intracellular signaling molecules (50).

Vascular NAD(P)H oxidase is regulated by many humoral factors, including cytokines, growth factors, and vasoactive agents (50). Physical factors, such as stretch, pulsatile strain, and shear stress, also stimulate NAD(P)H oxidase activation (32). Of particular importance, with respect to cardiovascular disease, is Ang II, which stimulates activation of NAD(P)H oxidase, increases expression of NAD(P)H oxidase subunits, and induces ROS production in cultured VSMCs, endothelial cells, adventitial fibroblasts, and intact arteries (14, 50). Oxidase activation occurs acutely by stimulation of intracellular signaling molecules (12, 93), which induce phosphorylation of p47phox and translocation of cytosolic subunits to gp91phox/p22phox to assemble the fully active enzyme. Chronic regulation of NADPH oxidase by Ang II involves *de novo* synthesis of NAD(P)H oxidase subunits (50, 93). These effects are mediated via AT_1 receptors (50, 62). Interestingly, ROS regulate AT_1 receptor gene expression, which in turn modulates ROS formation (59).

Subcellular processes linking Ang II/AT_1 to NAD(P)H oxidase and upstream signaling molecules regulating the oxidase in vascular cells have not been fully elucidated, but phospholipase D, phospholipase A, protein kinase C, c-Src, phosphatidylinositol 3-kinase, RhoA, and Rac have been demonstrated to be important in AT_1 signaling to NAD(P)H oxidase (50, 74, 88, 93).

In certain conditions, nitric oxide synthase (NOS), the enzyme primarily responsible for NO production, can also generate $\cdot O_2^-$. This occurs when there is a deficiency of substrate (arginine) or cofactor [tetrahydrobiopterin (BH_4)] (17, 56). These findings have led to the concept of "NOS uncoupling," where the activity of the enzyme for NO production is decreased in association with an increase in NOS-dependent $\cdot O_2^-$ formation. Endothelial NOS (eNOS) uncoupling has been demonstrated in various Ang II-dependent conditions, including atherosclerosis (97), diabetes (7), hyperhomocysteinemia (99), and hypertension (48). Gene transfer of GTP cyclohydrolase (GTPCH) I, the enzyme responsible for regenerating BH_4, restored arterial GTPCH I activity and BH_4 levels, reduced ROS, and improved endothelium-dependent relaxation and NO release in DOCA-salt hypertensive rats, in which endothelial dysfunction results from NAD(P)H-dependent oxidant excess (107). The potential role of uncoupling of NOS as a source of ROS in hypertension is also supported in human studies where increased endothelial $\cdot O_2^-$ production in vessels from diabetic and hypertensive patients is inhibited by sepiapterin, a precursor of BH_4 (36). The relative importance of NOS- versus NAD(P)H oxidase-mediated $\cdot O_2^-$ generation in cardiovascular disease probably relates, in part, to the magnitude of endothelial dysfunction, because most conditions in which $\cdot O_2^-$ is derived from uncoupled NOS are associated with marked endothelial dysfunction, such as in hyperlipidemia and diabetes.

Other Ang II-regulated enzymes capable of generating ROS in the vasculature are xanthine oxidase, cytochrome P450, mitochondrial respiratory chain enzymes, and phagocyte-derived myeloperoxidase (83). However, the contribution of these enzymes to vascular generation of ROS is relatively minor compared with NAD(P)H oxidase.

SIGNALING PATHWAYS AND MOLECULAR TARGETS OF ROS

ROS are involved in many Ang II-mediated signaling events, including activation of MAP kinases, tyrosine kinases, and transcription factors and deactivation of protein phosphatases (Fig. 1). In addition, growing evidence indicates that ROS influence Ca^{2+} signaling pathways and RhoA/Rho kinase cascades, important in Ang II regulation of vascular contraction/relaxation. The role of ROS in MAP kinase and tyrosine kinase/phosphatase signaling by Ang II is discussed elsewhere in this *Forum* issue and will only be briefly addressed here. The focus here will be on ROS, Ca^{2+}, and RhoA signaling by Ang II and implications in the regulation of vascular tone.

Protein tyrosine phosphatases and kinases

Protein-tyrosine phosphorylation is of major importance for cell proliferation, differentiation, migration, and transformation. The regulation of tyrosine phosphorylation is mediated by the antagonistic activity of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) (3). Because of their particular structure, PTPs are susceptible to oxidation and inactivation by ROS. The activation and inactivation of PTPs is regulated by extracellular signals, including Ang II (35). H_2O_2 plays a major role as a secondary messenger in this process (102).

Inactivation of PTPs is involved in oxidative stress-induced activation of several protein tyrosine kinases, such as the epidermal growth factor receptor (EGFR), insulin receptor, Lck, and Fyn (41). This is particularly important with respect to Ang II, which mediates many of its signaling events in vascular cells through EGFR transactivation (68). H_2O_2 has also been shown to regulate MAP kinases through inhibition of PTP activity of CD45, SHP-1, and HePTP (51). Thus, activation of vascular MAP kinases by Ang II may be mediated, in part, through redox-dependent inactivation of PTPs. However, this awaits further clarification.

Receptor and nonreceptor tyrosine kinases are also targets of oxidative stress. Exogenous H_2O_2 induces tyrosine phosphorylation and activation of platelet-derived growth factor receptor (PDGFR) and EGFR, probably due to ROS-mediated inhibition of dephosphorylation of PDGFR and EGFR by in-

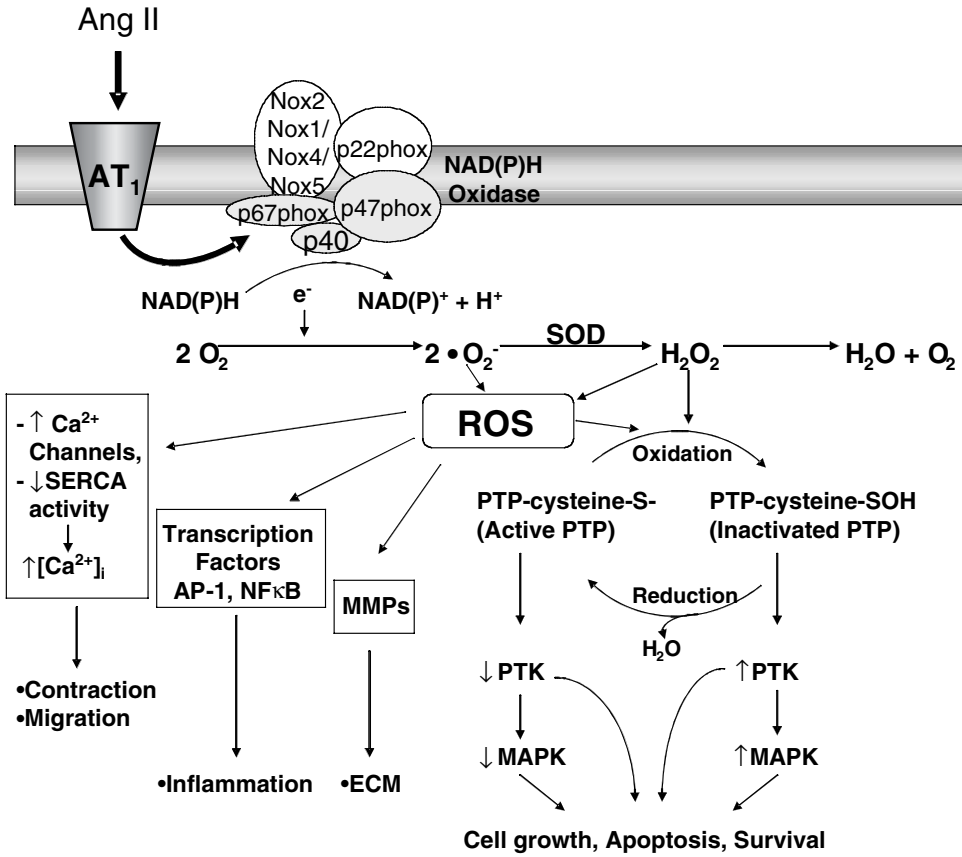

FIG. 1. Downstream targets of Ang II-generated ROS in vascular cells. Intracellular ROS influence the activity of protein tyrosine phosphatases (PTP) by modifying cysteine residues. Oxidation of cyteine residues to sulfenic acid by H_2O_2 renders PTPs inactive, whereas reduction renders PTPs active. Activated PTPs decrease protein tyrosine kinase (PTK) and mitogen-activated protein kinase (MAPK) activity, whereas inactivated PTPs have opposite actions. ROS also influence gene and protein expression by activating transcription factors, such as NFκB and AP-1. ROS stimulate Ca^{2+} channels and inhibit sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) leading to increased $[Ca^{2+}]_i$. ROS influence matrix metalloproteinases (MMPs), which modulate extracellular matrix protein (ECM) degradation. Activation of these redox-sensitive pathways results in many cellular responses. ↓, decreased effect, ↑, increased effect.

activation of membrane-associated PTPs (21). Oxygen intermediates, which are produced in response to tyrosine kinase receptor activation, are also involved in transactivation of PDGFR and EGFR by Ang II. This phenomenon involves c-Src and Ras (74). Under pathological conditions associated with oxidative stress, such as hypertension, atherosclerosis, and diabetes, ROS may directly activate cell surface receptors, thereby amplifying the process of $\cdot O_2^-$ generation. Nonreceptor tyrosine kinases such as Src, Janus kinase 2, STAT (signal transducers and activators of transcription), p21Ras, Pyk2, and Akt, all of which have been implicated in cardiovascular remodeling and vascular damage, are also regulated by ROS.

MAP kinases

MAP kinases participate in signal transduction classically associated with cell differentiation, cell growth, and cell death (60). Of the major mammalian MAP kinases, extracellular signal-regulated kinase (ERK) 1/2, p38 MAP kinase, and c-Jun N-terminal kinase (JNK) are the best characterized. ERK1/2, phosphorylated by MEK1/2 (MAP/ERK kinase), is a key growth signaling kinase, whereas JNK and p38 MAP kinase, phosphorylated by MEK4/7 and MEK3/6, respectively, influence cell survival, apoptosis, differentiation, and inflammation (60). ERK5, regulated by MEK5, is involved in protein synthesis, cell-cycle progression, and cell growth (60). ERK5 also plays an important role in protecting endothelial cells from apoptosis (61). Enhanced activation of vascular MAP kinases has been demonstrated in Ang II-dependent hypertension and seems to be a major mechanism contributing to vascular damage in hypertension and atherosclerosis (92, 103). MAP kinases are regulated by phosphorylation cascades (60). In addition, these kinases are strongly activated by ROS or by a mild oxidative shift of the intracellular thiol/disulfide redox state. Most studies examined effects of exogenous H_2O_2 to activate MAP kinases (5). There are relatively few reports of endogenous ROS regulating the MAP kinase cascade. In VSMCs, intracellular ROS are critical for Ang II-induced activation of p38 MAPK, JNK, and ERK5 (94). The importance of ROS in Ang II-mediated ERK1/2 activation in VSMCs remains unclear as studies have

demonstrated both redox-sensitive and redox-insensitive mechanisms (96, 98). Although MAP kinases are regulated by oxygen free radicals, they are probably not direct substrates of $\cdot O_2^-$ and H_2O_2. Upstream modulators, such as MEKs, tyrosine kinases, and phosphatases, may be direct targets. Inhibition of tyrosine phosphatases by thiol redox processes would lead to enhanced MAP kinase activity. In fact, decreased phosphatase activity has been linked to increased vascular ERK1/2 activation in hypertension (10).

Transcription factor activation and proinflammatory gene expression

Atherosclerosis, diabetes, and hypertension are increasingly recognized as inflammatory conditions in which cytokines, chemokines, and adhesion molecules play an important role in vascular inflammation and progression of atherosclerotic lesions. A major mechanism whereby proinflammatory gene expression is increased is through the activation of redox-sensitive transcription factors. Transcription factors, including nuclear factor-κB (NFκB), activator protein-1 (AP-1), c-Myb, Sp-1, p53, early growth response-1, and hypoxia-inducible factor-1, are directly activated by ROS (95). NFκB and AP-1 induce expression of proinflammatory genes, including monocyte chemotactic protein-1 (MCP-1), adhesion molecules, and interleukins (13, 67), that play a role in vascular inflammation associated with hypertension and atherosclerosis. Increased activation of redox-sensitive NFκB and AP-1 by Ang II has been demonstrated in VSMCs from spontaneously hypertensive rats (SHR) and in atherosclerotic lesions (66). Hence, this may be another mechanism whereby oxidative stress contributes to vascular damage.

INTERACTION OF ROS WITH Ca^{2+} IN Ang II SIGNALING

In addition to influencing cellular processes associated with cell growth and inflammation, ROS modulate intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), a major determinant of Ang II-induced vascular contraction/dilation (23) (Fig. 2). The role of intracellular Ca^{2+} and ROS as second messengers in the regulation of vascular signaling by Ang II is now well established (89). However, the interrelation of Ca^{2+} signals with ROS is less well known. Evidence supporting a role for ROS in the regulation of $[Ca^{2+}]_i$ in endothelial cells and VSMCs derives primarily from studies demonstrating that (a) exogenous addition of H_2O_2 or *tert*-butyl hydroperoxide alters Ca^{2+} transients, (b) exposure of cells to $\cdot O_2^-$-generating systems [*e.g.,* xanthine oxidase/hypoxanthine (XO/HX)] increases $[Ca^{2+}]_i$, and (c) NADPH stimulation induces intracellular Ca^{2+} mobilization.

Endothelial cells

Exposure of human umbilical endothelial cells to high concentrations of XO/HX results in a rapid transient $[Ca^{2+}]_i$ increase followed by a slow and sustained phase, similar to the signal pattern elicited by Ang II (20). The initial $[Ca^{2+}]_i$ peak is due in part to the rapid release of intracellular Ca^{2+} following receptor-mediated activation of phospholipase C and the consequent generation of inositol 1,4,5-trisphosphate ($InsP_3$). The sustained component results from Ca^{2+} influx (20). Exposure of endothelial cells to a lower $\cdot O_2^-$ concentration generated by XO/HX does not seem to alter basal $[Ca^{2+}]_i$, but significantly enhances agonist-stimulated Ca^{2+} signaling. This has been attributed to enhanced $InsP_3$-mediated Ca^{2+} mobilization (57).

In contrast to the $[Ca^{2+}]_i$-elevating effects of $\cdot O_2^-$, acute H_2O_2 stimulation attenuates agonist-stimulated Ca^{2+} signaling in endothelial cells (22). This is due to inhibition of intracellular Ca^{2+} mobilization by impaired extracellular Ca^{2+} influx. Lounsbury *et al.* described three phases of disturbed Ca^{2+} signaling by peroxide: (a) initially there is inhibition of agonist-stimulated Ca^{2+} influx with no change in resting $[Ca^{2+}]_i$, followed by (b) inhibition of agonist-induced reticular Ca^{2+} release with an increase in basal $[Ca^{2+}]_i$, and finally (c) a progressive increase in resting $[Ca^{2+}]_i$ and lack of a $[Ca^{2+}]_i$ response to agonist stimulation (52). Prolonged exposure of endothelial cells to peroxides results in increased endothelial $[Ca^{2+}]_i$, due in part to increased intracellular mobi-

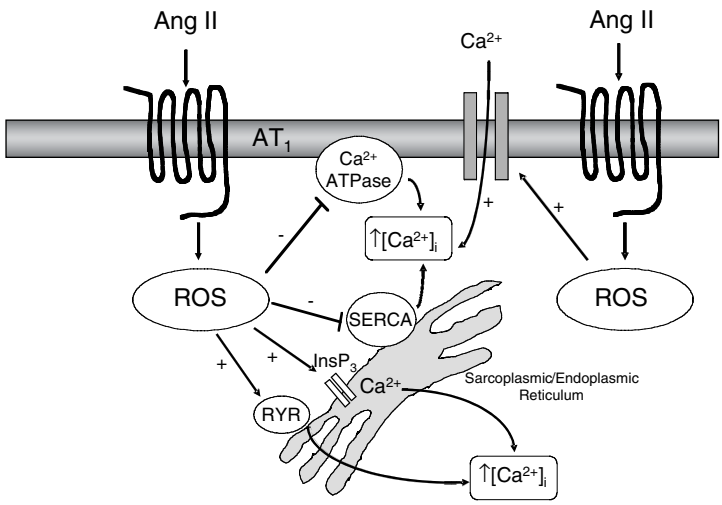

FIG. 2. Mechanisms of Ca^{2+} regulation by ROS in vascular cells. Changes in intracellular redox state increase $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx, by inducing inositol 1,4,5-trisphosphate ($InsP_3$)-induced $[Ca^{2+}]_i$ mobilization from intracellular stores, by stimulating ryanodine receptor (RYR) Ca^{2+}-release channels, and by inhibiting sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and plasma membrane Ca^{2+}-ATPase activity. +, stimulatory effect; −, inhibitory effect.

lization and in part to increased Ca^{2+} influx. Although earlier studies demonstrated opposite $[Ca^{2+}]_i$ effects of $\cdot O_2^-$ and H_2O_2 in endothelial cells, modest concentrations of $\cdot O_2^-$ and H_2O_2 (100 μmol/L) evoke changes similar to those induced by vasoactive agonists like Ang II, bradykinin, and histamine (39, 87).

VSMCs

Direct effects of ROS on Ca^{2+} signaling have also been observed in VSMCs. Most studies demonstrated a $[Ca^{2+}]_i$ stimulatory effect by exogenous ROS or XO/HX (23). These effects have been attributed to inhibition of Ca^{2+}-ATPase and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and to activation of Ca^{2+} channels (33, 79). SERCA normally pumps Ca^{2+} against its concentration gradient into the sarcoplasmic reticulum lumen, whereas plasma membrane Ca^{2+}-ATPase pumps Ca^{2+} across the cell membrane. Hence, inactivation of these ATPases by ROS results in accumulation of intracellular Ca^{2+} with consequent increase in cytosolic $[Ca^{2+}]_i$. Mechanisms whereby ROS inhibit SERCA are probably through irreversible oxidation of sulfhydryl groups or by direct effect on ATP-binding sites (71). SERCA inhibition by oxidation of sulfhydryl groups is supported by the ability of reducing agents and cysteine to prevent inhibition, the decline in sulfhydryl content of oxidized sarcoplasmic reticulum, and the ability of sulfhydryl-binding agents to inhibit Ca^{2+}-ATPase activity. In deendothelialized coronary arteries, damage to SERCA by ROS is associated with blunted contractile responses to Ang II or the SERCA pump inhibitors thapsigargin and cyclopiazonic acid (34). This effect is more pronounced in small arteries where there is a high density of SERCA pumps.

In addition to SERCA pumps, transport proteins are targets of ROS. ROS interact with various ion transport proteins, including (a) ion channels, such as Ca^{2+} channels (voltage-sensitive L-type Ca^{2+} channels, dihydropyridine receptor voltage sensors, ryanodine receptor Ca^{2+}-release channels, and D-*myo*-inositol 1,4,5-trisphosphate receptor Ca^{2+}-release channels), K^+ channels (Ca^{2+}-activated K^+ channels), Na^+ channels, and Cl^- channels; (b) ion pumps, such as Na^+, K^+-ATPase and Ca^{2+}-ATPase; (c) ion exchangers, such as the Na^+/Ca^{2+} exchanger and the Na^+/H^+ exchanger; and (d) ion cotransporters, such as K^+/Cl^- and $Na^+/K^+/Cl^-$ cotransporters (46). As all of these transporters are regulated, to varying degrees, by Ang II (82), it is possible that Ang II-generated ROS play a role in transmembrane ion transport in Ang II-stimulated vascular cells. We recently demonstrated that H_2O_2 and $\cdot O_2^-$ stimulate Ca^{2+} influx in VSMCs through L-type and T-type Ca^{2+} channels (82). Interestingly, H_2O_2-induced Ca^{2+} influx was increased in cells from SHR, an Ang II-sensitive model of hypertension, and this was attributed to increased expression of vascular L-type Ca^{2+} channels (82). Mechanisms of ROS-induced modifications in ion transport pathways involve oxidation of sulfhydryl groups located on the ion transport proteins, peroxidation of membrane phospholipids, and inhibition of membrane-bound regulatory enzymes (ATP) (46) (Table 1).

Redox-dependent regulation of $[Ca^{2+}]_i$ may influence the nature and efficiency of Ca^{2+} signaling. In light of this, it has been demonstrated that application of agonist stimulation together with ROS-mediated oxidation induces long-lasting potentiation of subsequent Ca^{2+} signaling (105). This is particularly relevant to Ang II-mediated redox signaling in VSMCs, because Ang II induces ROS formation, which in turn both stimulates Ca^{2+} signaling and up-regulates AT_1 receptors. Up-regulated redox-dependent AT_1 signaling further stimulates Ca^{2+} signaling pathways, thereby amplifying the Ang II-stimulated redox-regulated Ca^{2+} signaling pathway (59) (Fig. 3).

TABLE 1. MOLECULAR MECHANISMS WHEREBY ROS INFLUENCE Ca^{2+} TRANSPORT SYSTEMS

1. Oxidation of sulfhydryl groups located on the ion transport protein
2. Peroxidation of membrane phospholipids
3. Inhibition of membrane-bound regulatory enzymes
4. Oxidative phosphorylation of ion transport proteins
5. Modification of ATP levels

In addition to regulating vascular contractile machinery through Ca^{2+}-dependent pathways, it is also possible that ROS influence vascular tone through RhoA/Rho kinase cascades. RhoA is a low-molecular-weight guanosine triphosphatase that is regulated by Ang II (58). RhoA activation leads to stimulation of Rho kinase, promoting cell contraction via phosphorylation of the myosin-binding subunit of myosin light chain phosphatase (thereby inhibiting phosphatase activity) (27) (Fig. 4). Increased vascular reactivity in hypertension may be due to increased Ca^{2+} sensitization due to RhoA/Rho kinase up-regulation (42, 44, 54). NADPH oxidase-generated $\cdot O_2^-$ has been implicated in these processes as demonstrated in a recent study where long-term inhibition of Rho kinase suppressed Ang II-induced cardiovascular hypertrophy in rats through NADPH oxidase-sensitive mechanisms (37). ROS may influence RhoA/Rho kinase by modulating guanine nucleotide exchange factor activation, and ROS formation itself seems to be regulated by RhoA-dependent mechanisms (31, 37, 49, 85).

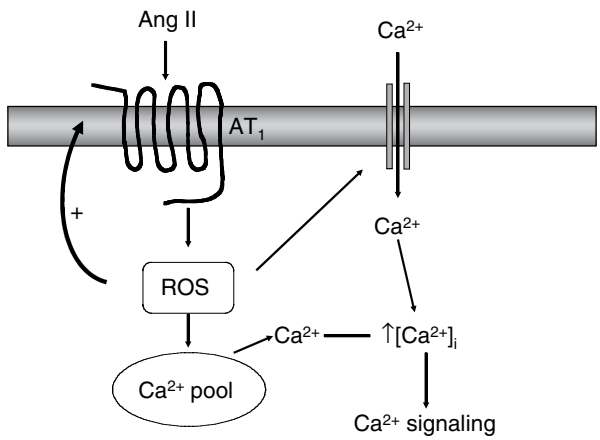

FIG. 3. Putative mechanisms whereby Ang II-induced redox-dependent Ca^{2+} signaling is amplified. Ang II, through AT_1 receptors, induces ROS formation, which in turn increases $[Ca^{2+}]_i$ and up-regulates AT_1 receptors. This circuitous process could result in amplification of redox signaling by Ang II, as indicated by the "+" sign.

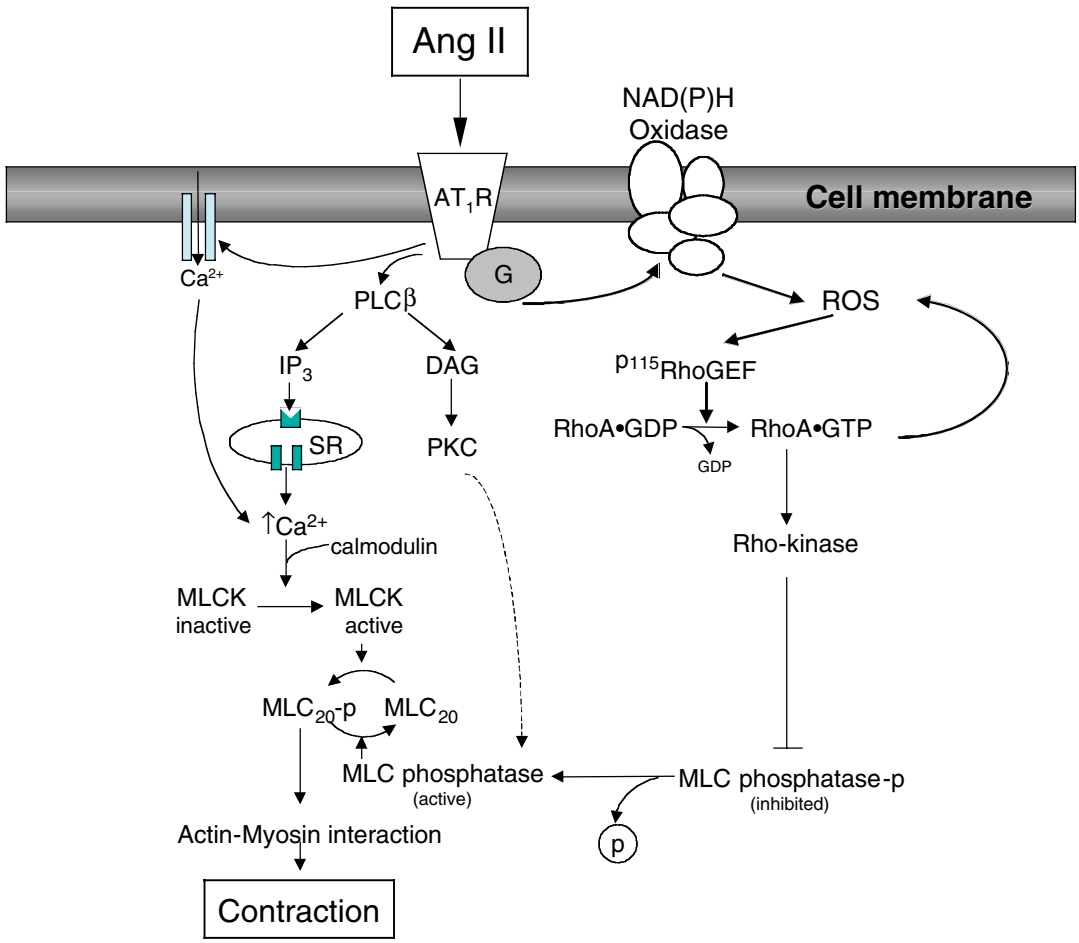

FIG. 4. RhoA/Rho kinase mechanisms involved in Ang II-induced VSMC contraction. NAD(P)H oxidase-derived ROS influence RhoA-dependent signaling. RhoA in turn may influence ROS production. DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; IP_3, inositol trisphosphate; MLC, myosin light chain; p, phosphorylated; PLC, phospholipase C; SR, sarcoplasmic reticulum.

ROS, Ang II, AND VASCULAR TONE

Studies as early as the 1980s reported that H_2O_2 is a potent vasodilator in cerebral arteries (45). More recent investigations suggested that H_2O_2 induces vasoconstriction in various vascular beds, including rat mesenteric arteries, rat aorta, porcine pulmonary arteries, and canine cerebral arteries. $\cdot O_2^-$ has also been shown to induce vasoconstriction in certain vascular beds and to reduce relaxation to endothelium-dependent vasodilators (65). This may be either the result of NO scavenging or the result of a direct Ca^{2+}-dependent contractile effect on vascular smooth muscle (43). However, not all reports are in agreement with these findings. Indeed studies of the effects of oxygen free radicals on vascular tone often report contradictory results. In studies performed in isolated human and porcine coronary or femoral arteries in organ baths, $\cdot O_2^-$ did not play a role in Ang II-induced vasoconstriction and, if anything, resulted in vasodilation through H_2O_2 formation (73). These results support those found in cerebral arteries (100), coronary arteries (9), and pulmonary arteries (9, 100), but are in contrast with those of others who demonstrated that H_2O_2 is a critical intracellular metabolite in vascular contractile responses to Ang II (84).

Mechansims underlying ROS-induced vasoconstriction or dilation probably involve differential regulation of Ca^{2+} signaling pathways. ROS increase VSMC $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx, increasing $InsP_3$-mediated Ca^{2+} mobilization, and inhibiting SERCA pump activity. Increased $[Ca^{2+}]_i$ induces actin–myosin complex formation and consequent contraction. In the endothelium, increased ROS-mediated $[Ca^{2+}]_i$ may stimulate Ca^{2+}-dependent NOS activity, resulting in NO production and consequent vasodilation. It is also possible that elevated endothelial $\cdot O_2^-$ could quench NO, resulting in $ONOO^-$ formation, described as a weak vasodilator. Hence, $\cdot O_2^-$ could have both vasoconstrictory and vasodilatory properties, depending on the primary location, species, and concentration of the free radical generated by Ang II. To support further a relationship between ROS, Ang II, and vascular control, Torrecillas *et al.* demonstrated that Ang II-induced aortic contraction is inhibited by catalase (84), and we recently re-

ported that H_2O_2-induced contractile and $[Ca^{2+}]_i$ responses are enhanced in resistance arteries from SHR, an Ang II-dependent model of hypertension (82).

H_2O_2 also has a dual vasoactive effect. It induces vasodilation via stimulation of prostaglandins, by cyclic GMP mechanisms, or through activation of Ca^{2+}-dependent K^+ channels. H_2O_2 elicits vasoconstriction by stimulating Ca^{2+} influx and increasing VSMC $[Ca^{2+}]_i$. In the endothelium, H_2O_2 has been considered to be an important endothelium-derived hyperpolarizing factor (55).

It is evident that ROS are capable of inducing actions that could promote both vasodilation and vasoconstriction. Major factors underlying the differential vascular responses to activated oxygen metabolites could relate to the blood vessel studied, the presence or absence of the endothelium, the concentration and species of free radical studied, and the compartment in which $\cdot O_2^-$ or H_2O_2 predominates (86) (Fig. 5). Vascular $\cdot O_2^-$, which is generally cell membrane-impermeable, is located primarily intracellularly in adventitial fibroblasts and VSMCs, whereas H_2O_2 is easily diffusible and migrates to the extracellular milieu or traverses the vascular wall to reach the endothelium. Accordingly, increased VSMC $\cdot O_2^-$ may indeed lead to elevated $[Ca^{2+}]_i$ and consequent vasoconstriction, whereas predominantly increased endothelial or extracellular H_2O_2 would promote vasodilation. The exact role of these processes in Ang II-regulated vascular tone awaits clarification.

ROS, VASCULAR GROWTH, AND INFLAMMATION

In vascular damage when oxidative stress is increased, redox-sensitive growth processes may lead to accelerated proliferation and hypertrophy, further contributing to vascular injury and remodeling (53). ROS also induce apoptosis and differentiation under certain circumstances. This differential response appears to relate to the specific species generated, the concentration of ROS, and the cellular localization of ROS. At high concentrations (>100 μmol/L), H_2O_2 and $ONOO^-$ are proapoptotic and induce anoikis (cell detachment and shedding), whereas at lower concentrations they stimulate growth and differentiation (19).

ROS also modulate vascular structure in cardiovascular disease by increasing deposition of extracellular matrix proteins, such as collagen and fibronectin. $\cdot O_2^-$ and H_2O_2 influence activity of vascular matrix metalloproteinases 2 and 9, which promote degradation of basement membrane and elastin, respectively (63). Redox-sensitive inflammatory processes, including expression of proinflammatory molecules, such as MCP-1, osteopontin, and interleukin-6, expression of adhesion molecules, including vascular cell adhesion molecule-1 and intracellular adhesion molecule-1, lipid peroxidation, and cell migration, further contribute to vascular remodeling in hypertension (77).

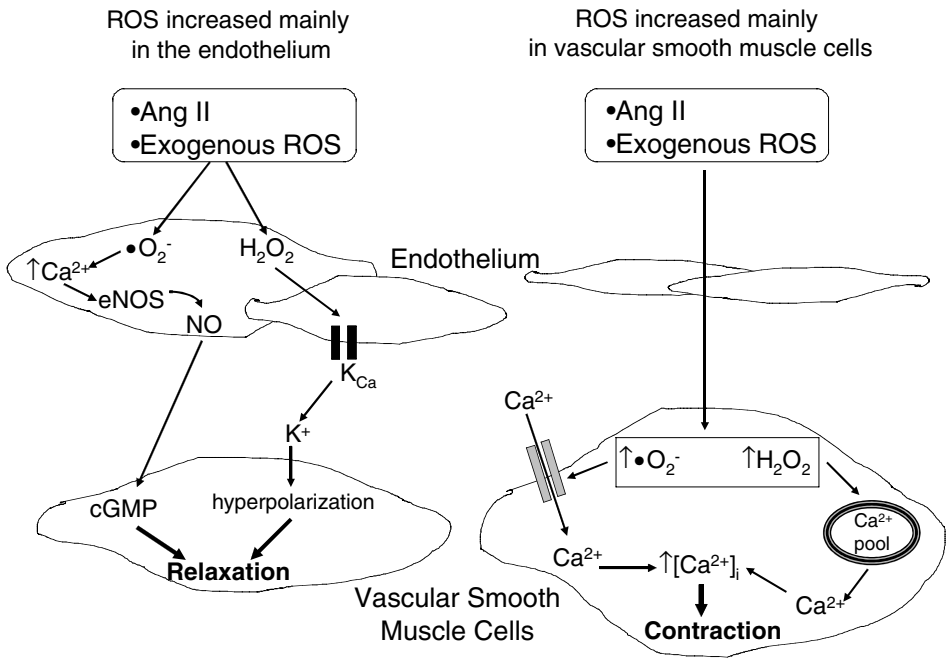

FIG. 5. Possible mechanisms whereby $\cdot O_2^-$ and H_2O_2 mediate constriction and/or dilation in vessels. Increased bioavailability of $\cdot O_2^-$ and H_2O_2 in VSMCs induces Ca^{2+} influx through activated Ca^{2+} channels and enhanced Ca^{2+} mobilization from sarcoplasmic reticular stores, resulting in increased $[Ca^{2+}]_i$ and consequent contraction. Increased bioavailability of endothelial ROS causes activation of K_{Ca} channels, resulting in hyperpolarization. Endothelial $[Ca^{2+}]_i$ elevation induced by $\cdot O_2^-$ increases eNOS activity leading to NO production, which in the presence of $\cdot O_2^-$, forms $ONOO^-$. NO relaxes VSMCs through cyclic GMP (cGMP)-dependent mechanisms. Depending on the species, concentration, cellular location, absence or presence of endothelium, and agonist, ROS appear to induce differential vasoactive responses.

Oxygen radicals induce endothelial permeability with extravasation of plasma proteins and other macromolecules, and recruitment of inflammatory proteins and cells, which also impair endothelial function and aggravate vascular damage (2). Peripheral polymorphonuclear leukocytes, which generate $\cdot O_2^-$, participate in oxidative stress and inflammation in patients with hypertension. The coexistence of an inflammatory reaction with oxidative stress induces endothelial dysfunction. Many of the redox-sensitive vascular changes that occur in hypertension also exist in atherosclerotic vessels.

CONCLUSIONS

ROS are produced in the vessel wall in a controlled and tightly regulated manner. Ang II is a potent inducer of NADPH-driven generation of ROS in the endothelium, vascular media, and adventitia. $\cdot O_2^-$ and H_2O_2 have important signaling properties, mainly through oxidative modification of proteins and activation of transcription factors. In addition to influencing tyrosine kinases, protein phosphatases, and MAP kinases, ROS modulate intracellular Ca^{2+} signaling in the endothelial cells and VSMCs. ROS increase $[Ca^{2+}]_i$ by stimulating $InsP_3$-mediated mobilization of intracellular Ca^{2+}, by increasing cytosolic Ca^{2+} accumulation through inhibition of SERCA, and by stimulating Ca^{2+} influx through voltage-dependent Ca^{2+} channels. ROS also influence contractile processes by stimulating RhoA/Rho kinase cascades. These redox-dependent signaling events may influence Ang II-regulated vascular contraction/relaxation and tone. In cardiovascular diseases such as hypertension, atherosclerosis, hyperlipidemia, and ischemia–reperfusion injury, dysregulation of NAD(P)H oxidase, NOS, and xanthine oxidase by Ang II results in increased ROS formation and oxidative stress. ROS contribute to vascular injury by increasing vascular tone through Ca^{2+}-dependent and RhoA/Rho kinase signaling pathways, and by promoting VSMC growth, extracellular matrix protein deposition, activation of matrix metalloproteinases, and inflammation. As ROS play a central role in many Ang II-mediated signaling events, they could be critically involved in cardiovascular disease processes associated with activation and dysregulation of the renin–angiotensin system.

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ABBREVIATIONS

Ang II, angiotensin II; AP-1, activator protein-1; AT_1, angiotensin II type 1 receptor; BH_4, tetrahydrobiopterin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; EC-SOD, extracellular superoxide dismutase; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; GTPCH I, GTP cyclohydrolase I; H_2O_2, hydrogen peroxide; $InsP_3$, inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MEK1/2 kinase, mitogen-activated protein/extracellular signal-regulated kinase kinase; NFκB, nuclear factor κB; NO, nitric oxide; NOS, nitric oxide synthase; Nox, NAD(P)H oxidase; NoxA1, Nox activator 1; NoxO1, Nox organizer 1; $\cdot O_2^-$, superoxide anion; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite; PDGFR, platelet-derived growth factor receptor; phox, phagocyte oxidase; PTP, protein tyrosine phosphatase; redox, reduction–oxidation; ROS, reactive oxygen species; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; VSMC, vascular smooth muscle cell; XO/HX, xanthine oxidase/hypoxanthine.

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