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FORUM REVIEW ARTICLE

Reactive Oxygen Species, Vascular Noxs, and Hypertension: Focus on Translational and Clinical Research

Augusto C. Montezano and Rhian M. Touyz

Abstract

Significance: Reactive oxygen species (ROS) are signaling molecules that are important in physiological processes, including host defense, aging, and cellular homeostasis. Increased ROS bioavailability and altered redox signaling (oxidative stress) have been implicated in the onset and/or progression of chronic diseases, including hypertension. **Recent Advances:** Although oxidative stress may not be the only cause of hypertension, it amplifies blood pressure elevation in the presence of other pro-hypertensive factors, such as salt loading, activation of the renin-angiotensin-aldosterone system, and sympathetic hyperactivity, at least in experimental models. A major source for ROS in the cardiovascular-renal system is a family of nicotinamide adenine dinucleotide phosphate oxidases (Noxs), including the prototypic Nox2-based Nox, and Nox family members: Nox1, Nox4, and Nox5. **Critical Issues:** Although extensive experimental data support a role for increased ROS levels and altered redox signaling in the pathogenesis of hypertension, the role in clinical hypertension is unclear, as a direct causative role of ROS in blood pressure elevation has yet to be demonstrated in humans. Nevertheless, what is becoming increasingly evident is that abnormal ROS regulation and aberrant signaling through redox-sensitive pathways are important in the pathophysiological processes which is associated with vascular injury and target-organ damage in hypertension. **Future Directions:** There is a paucity of clinical information related to the mechanisms of oxidative stress and blood pressure elevation, and a few assays accurately measure ROS directly in patients. Such further ROS research is needed in humans and in the development of adequately validated analytical methods to accurately assess oxidative stress in the clinic. *Antioxid. Redox Signal.* 20, 164–182.

Introduction

HYPERTENSION IS A multifactorial, complex disorder, involving many organ systems (41, 215). Factors that are important in the development of hypertension include activation of the sympathetic nervous system, up-regulation of the renin-angiotensin-aldosterone system, altered G protein-coupled receptor signaling, and inflammation (81, 214). Recent studies also implicate a role of the immune system in hypertension (82, 175). Common to these processes is oxidative stress (excess levels of oxidants over antioxidants), due, mainly, to increased production of reactive oxygen species (ROS), decreased nitric oxide (NO) levels, and reduced antioxidant capacity in the cardiovascular, renal, and central nervous systems (Fig. 1) (208, 220).

ROS, including superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), were originally considered harmful metabolic by-

products of mitochondrial energetics and cell metabolism. However, ROS are now recognized to have important physiological functions through their modulation of the redox state of signaling molecules (52). ROS influence many signaling pathways, including mitogen-activated protein kinases (MAPK), tyrosine kinases, Rho kinase, transcription factors (NF κ B, AP-1, and HIF-1), and protein tyrosine phosphatases that impact cardiovascular, renal, and neural cell function (26, 134, 158, 161, 189). ROS increase intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) through ion channel activation and up-regulate protooncogene and proinflammatory gene expression and activity (40, 199). Uncontrolled generation of ROS promotes oxidative stress and consequent damage to DNA, proteins, and lipids, leading to cell injury and cytotoxicity (96, 115, 118). Physiologically, ROS regulate cellular processes such as differentiation, proliferation, apoptosis, cell cycle, migration, secretion, cytoskeletal organization, activation of

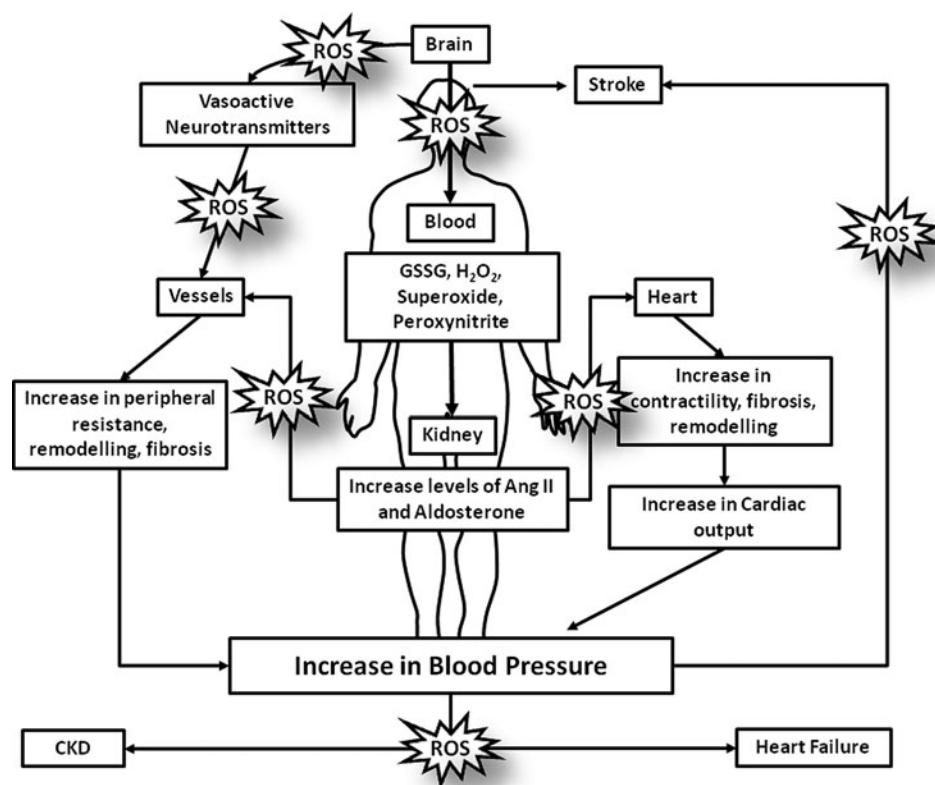


FIG. 1. Systemic oxidative stress in humans: implications in hypertension. Pathophysiological levels of reactive oxygen species (ROS) will lead to a series of deleterious effects in the human body. In the brain, ROS increase the production and release of vasoactive neurotransmitters, which, in turn, will increase or decrease vascular resistance, an effect that influences blood pressure. In the kidney, ROS increases the production and the release of many vasoactive agents, including angiotensin II (Ang II) and aldosterone, which, in turn, influence cardiac, vascular, and renal function through redox-sensitive processes. ROS influence processes that are involved in vascular remodeling, endothelial dysfunction, fibrosis, and inflammation, which are characteristic features in hypertension. Moreover, in the heart, ROS have been linked to increased contractility, fibrosis and cardiac remodeling; reflecting in an increase in cardiac output and blood pressure. All of these ROS-related effects impact the development of hypertension and its target organ damage, predisposing to stroke, heart failure, and chronic kidney disease (CKD).

transcription factors, and gene expression (71, 98, 212). In the vascular system, ROS play a physiological role in controlling endothelial function and vascular tone and a pathophysiological role in processes underlying endothelial dysfunction, hyperactivity, inflammation, and vascular remodeling in cardiovascular diseases, including hypertension (4, 19, 200, 218).

ROS are usually produced from cellular respiration and metabolic processes as byproducts *via* activation of enzymes such as xanthine oxidoreductase, uncoupled NO synthase (NOS), and mitochondrial respiratory enzymes (2, 65, 150, 186) (Fig. 2). In addition, ROS are produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox). Noxs, of which there are seven isoforms, and that function primarily as ROS-generating enzymes, are an important source of $O_2^{\bullet-}$ and H_2O_2 in the cardiovascular system (209). When dysregulated Noxs play a role in increased ROS production, it leads to endothelial dysfunction and vascular remodeling in hypertension.

The relationship between ROS and blood pressure was first suggested in the 1960s (170), but it was in the early 2000s that this association was explored in detail when it was shown that angiotensin II (Ang II)-induced hypertension in rats increases

vascular ROS production *via* non-phagocytic NADPH oxidase activation (162). Most experimental models of hypertension exhibit some degree of oxidative stress (48, 67, 93, 95, 236). Moreover, mice with reduced antioxidant enzyme systems and those deficient in NADPH oxidase have higher blood pressures than those with intact systems. Based on extensive experimental data, it has been suggested that oxidative stress is causally associated with hypertension, at least in animal models.

In clinical medicine, the direct relationship between ROS and hypertension is not convincing, and there is still no definitive proof that oxidative stress is a direct cause of hypertension in humans. In fact, despite extensive data in the literature implicating a role for ROS and oxidative stress in many chronic diseases, such as cardiovascular disease, diabetes, cancer, and kidney disease, there are very few clinical conditions that are directly due to altered ROS levels. These include vitiligo, neurodegenerative diseases, and progeria (67, 99, 117, 145, 172, 216). With regard to clinical hypertension, most studies examining ROS are based on associations between plasma or urine markers of oxidative stress and blood pressure. Biomarkers of cell damage due to systemic oxidative stress, such as plasma thiobarbituric

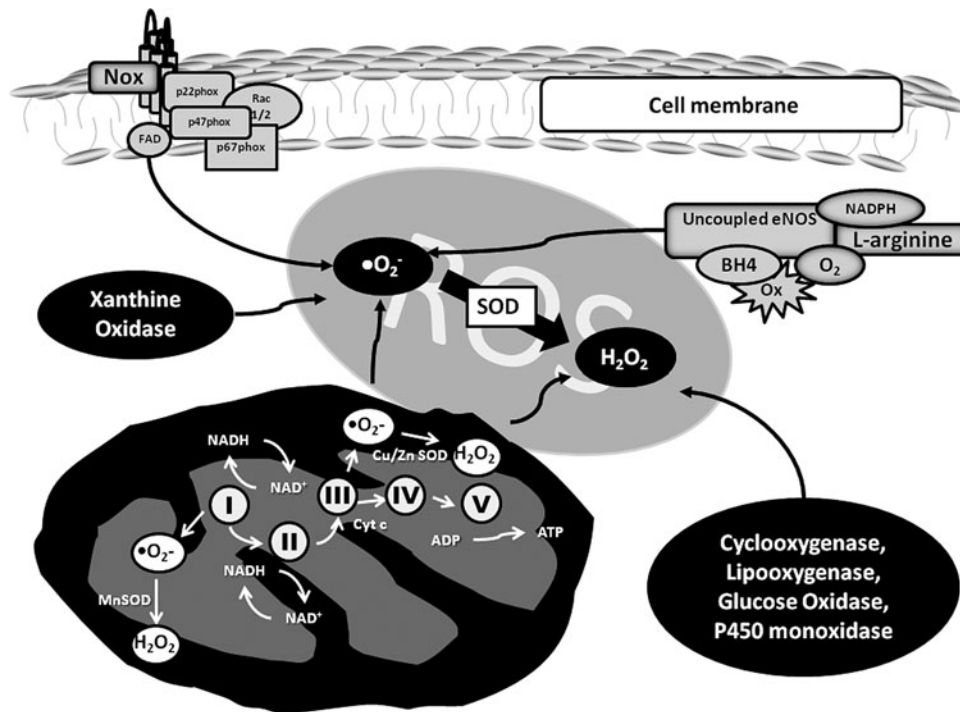


FIG. 2. Sources of ROS in cells. ROS production is regulated by many tightly controlled systems. ROS are produced either as a byproduct of an enzyme or as the main product of nicotinamide adenine dinucleotide phosphate oxidase (Nox) activity. Except for Nox, which has as its main function the formation of ROS, the other enzymes generate ROS as byproducts of enzymatic activity. Uncoupled endothelial nitric oxide synthase (eNOS): In the absence of L-arginine or tetrahydrobiopterin, the electrons in the enzyme reduce molecular oxygen to superoxide, instead of NO. Damaged or dysfunctional mitochondria generate increased amounts of superoxide, creating a state of redox imbalance and consequent oxidative stress. Nox: The Nox family comprises seven isoforms, where the generation of ROS is the main and only function. For these enzymes to be biologically active, they require a number of subunits to assemble at the membrane in order to form ROS. Other enzymes that generate ROS are xanthine oxidase, cyclooxygenase, lipoxygenase, glucose oxidase, and P450 mono-oxidases.

acid-reactive substances (TBARS) and 8-epi-isoprostanes, are elevated in patients with hypertension (45, 116). Antioxidant capacity and levels of antioxidant vitamins and enzymes have been shown to be reduced in patients with hypertension (66, 107). Such studies between hypertension and oxidative stress are purely correlative and are far from proving cause.

Hence, despite the notion that oxidative stress underlies hypertension, there is still little solid evidence for this at the clinical level. Possible reasons relate to a paucity of information on molecular mechanisms of ROS biology in human tissue, lack of adequate methods to evaluate ROS in the clinical setting, and inappropriately designed clinical trials to evaluate the effects of antioxidant therapy on hypertension. It is also possible that, although oxidative stress may be important in pathophysiological mechanisms that are associated with cardiac, vascular, renal, and neural dysfunction and remodeling, which could influence blood pressure (Fig. 1), it may not be an important primary causative factor in the pathogenesis of hypertension in humans. These themes will be developed and discussed in the present review. While it is appreciated that ROS have an impact on many systems that influence blood pressure regulation and development of hypertension, here, we will focus on the role of vascular ROS in hypertension, highlighting translational research and clinical studies.

Production of ROS in the Vascular System: Spotlight on Noxs

Noxs are transmembrane-associated proteins that transfer electrons across membranes, such that the final electron acceptor is O_2 and $O_2^{\bullet-}$ is generated (113). The mammalian Nox family comprises seven isoforms: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2 (16, 113, 182). Nox1, 2, 4, and 5 are expressed in multiple tissues; whereas Nox3 and the Duoxs are more tissue specific, with Nox3 expressed in the inner ear and Duox1/2 in the thyroid gland. All Noxs are transmembrane proteins that have a core catalytic subunit (Nox) and a number of regulatory subunits (Fig. 3).

The prototypical Nox is Nox2 (phagocytic NADPH oxidase) and comprises five subunits: p47phox, p67phox, p40phox, which are cytosolic regulatory proteins, p22phox, a membrane regulatory protein, and gp91phox, which is the catalytic subunit (12). Nox2 is activated when the regulatory subunit p47phox is phosphorylated, complexes with p67phox and p40phox, and translocates to the cell membrane to interact with p22phox and Nox2 to assemble the active oxidase, which then transfers electrons from the substrate (NADPH) to O_2 forming $O_2^{\bullet-}$ (119).

Nox1, Nox2, Nox4, and Nox5 have been identified in the vascular system (24, 111). Hyperactivation of Noxs leads to excessive ROS production that disrupts redox networks,

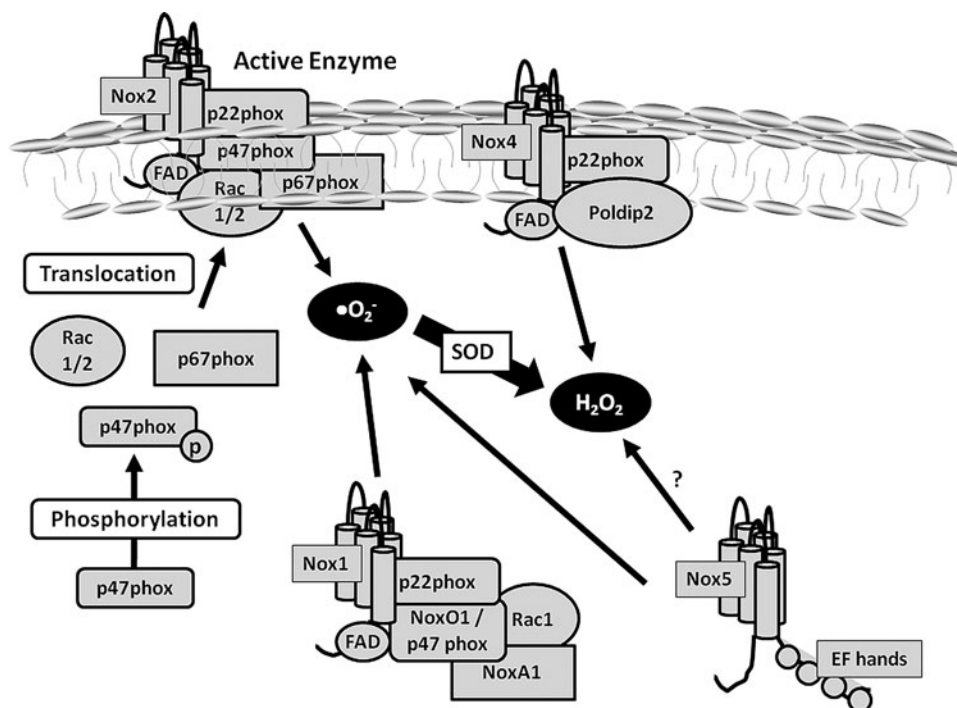


FIG. 3. Nox activation in the cardiovascular system. Nox1, Nox2, Nox4, and Nox5 are members of the Nox family that are expressed in the cardiovascular system. Nox2 is the classical Nox that is primarily characterized in leukocytes and related to host defence responses. In order to be activated, cytosolic subunits (p47phox, p67phox, and Rac 1/2) should translocate from the cytosol to the membrane and bind to the other two membrane-bound subunits, Nox2 and p22phox. ROS is generated once the whole enzyme complex is formed. Nox1 activation is similar to Nox2, but it depends on the cytosolic subunits NoxO1 (a homologue of p47phox) and NoxA1 (a homologue of p67phox) in order to be active. Nox4 only depends on p22phox in order to be active, is constitutively activated, and ROS production is regulated by Poldip2. Nox5 does not depend on any other subunits to be activated. It has four EF hands, which are binding sites for calcium. Nox5 activity is regulated not only by calcium changes in the cells, but also by calmodulin and by phosphorylation of kinases.

which is usually regulated by thiol-dependent antioxidant systems. This leads to oxidative stress, triggering molecular processes, which, in the vasculature, contributes to vascular injury and inflammation. The Noxs have been extensively reviewed (7, 16, 53, 82, 113, 160), and only an overview of recent developments is discussed here, focusing on the most recently characterized isoform, Nox5.

Nox1

Nox1 is abundant in colon epithelium, but is also found in endothelium, smooth muscle, fibroblasts, cardiomyocytes, and microglia (151). It requires p22phox, p47phox (or its homologue NoxO1 [Nox organizer 1]), and p67phox (or its homologue NoxA1 [Nox activator 1]) for its activity. Nox1-derived $O_2^{\bullet-}$ is increased in a stimulus-dependent manner, involving complex interactions between regulatory subunits and the redox chaperone protein disulfide isomerase (54, 58).

In cultured endothelial and vascular smooth muscle cells (VSMCs), Nox1 is up-regulated by mechanical factors (shear stress), vasoactive agents (Ang II, aldosterone), and growth factors (epidermal growth factor, platelet-derived growth factor [PDGF]) (128, 159). Induction of Nox1 by Ang II may involve mitochondria, possibly through a Ca^{2+} -dependent mechanism (163). Nox1 is also activated by H_2O_2 in VSMCs. Exogenous H_2O_2 , which may enter cells *via* aquaporins, increased Nox1-derived $O_2^{\bullet-}$ generation, leading to hypertrophy, a process that is mediated *via* Ask1 (3).

Nox1 plays a role in VSMC migration, proliferation, and extracellular matrix production, effects that are mediated by cofilin (219). It has also been implicated in blood flow regulation through a mechanism involving thrombospondin-1 (TSP1) and CD47 (46). Blockade of CD47 and Nox1 gene silencing *in vivo* in rats improved TSP1-induced impairment of tissue blood flow after ischemia reperfusion. These novel data suggest a highly regulated process of ROS stimulation and blood flow regulation promoted through direct TSP1/CD47-mediated activation of Nox1 and define a regulatory role for TSP1 *via* CD47, Nox1, and ROS in tissue injury and reperfusion.

Nox1 expression/activity is increased in the vasculature in models of cardiovascular disease, including hypertension, atherosclerosis, diabetes, and hypercholesterolemia (50, 190). Nox1-deficient mice have decreased expression of aortic AT_1R (130), which may contribute to blunted pressor actions of Ang II infusion in these mice. Although there is extensive experimental data suggesting a role for Nox1 in cardiovascular disease, there is little information in humans, although expression of Nox1 and NoxA1 is increased in human atherosclerotic vessels (152).

Nox2

Nox2 is not only the catalytic subunit of the respiratory burst Nox in phagocytes, but it is also expressed in vascular cells (210), where it localizes in the cell membrane, as well as

with the cytoskeleton, lipid rafts/caveolae, and the perinuclear compartment. The Nox2 gene is inducible and is highly regulated by Ang II and stretch (51, 149). Vascular Nox2, derived from resident macrophages or vascular cells, is up-regulated in experimental hypertension, atherosclerosis, ischemia-reperfusion injury, and neointimal formation (74, 211). Nox2 is also implicated in stroke in experimental models (34).

Nox4

Nox4 is found in vascular cells, fibroblasts, adipocytes, hepatocytes, and renal cells (64, 84, 180). In VSMCs, Nox4 colocalizes with p22phox and vinculin in focal adhesions and plays a role in cell migration, proliferation, and cell differentiation (35). Nox4 has been identified in the endoplasmic reticulum, mitochondria, and nucleus, and it is constitutively active and regulated mainly by its level of expression (8). Nox4 appears to generate H_2O_2 , although the primary product is probably $O_2^{\bullet-}$, which is rapidly dismutated to H_2O_2 (202, 232). Nox4 contributes to basal ROS production through its constitutive activity and to increased ROS generation when induced by Ang II, glucose, tumor necrosis factor α , and growth factors (90, 127). Recent studies have identified a 28-kDa Splice Variant of Nox4 located in the nucleus of vascular cells, which may be important in pathophysiologic effects through modulation of nuclear signaling and DNA damage (9). The pathological role of Nox4 is unclear, although it has been implicated in hypertension, atherosclerosis, and cardiovascular and renal complications of diabetes and in remodeling of pulmonary arteries in pulmonary hypertension (181, 206). Nox4-derived ROS has also been suggested in cellular senescence and aging (104) and in insulin-mediated differentiation of adipocytes (177). Recent studies demonstrated that Nox4 may actually have protective effects, possibly through Nox4-derived H_2O_2 , which may act as a vasodilator in some vascular beds (164, 238). This could explain why mice with targeted endothelial Nox4 overexpression have lower blood pressure and improved endothelium-dependent vasodilation *versus* wild-type controls (164).

Nox5

Nox5 is the most recently identified Nox and is unique: It is Ca^{2+} sensitive, possesses a calmodulin-like domain with Ca^{2+} binding sites, and does not require any Nox subunits for its activity (62, 92, 138, 185). Nox5 was first identified in testes and spleen and, more recently, in vascular cells. 5 splice variants have been identified: α , β , δ , γ , and ϵ (157). While the functional roles for each of these variants have yet to be discovered, all share a number of features common to all Noxs, including six transmembrane spanning domains, two groups of heme-spanning histidines, an NADPH-binding motif, and an FAD-binding domain. Vascular Nox5 is activated by PDGF, Ang II, and ET-1 and it involves ERK1/2, PI(4, 5)P₂, PKC, and c-Abl (17, 137, 155). Hsp90 binds to and regulates Nox5 protein stability (56, 126, 33). In human endothelial cells (ECs), siRNA-mediated Nox5 knockdown reduced Ang II stimulated ERK1/2 activation, but not that of p38 MAPK or JNK (56). Nox5 generates ROS in response to increases in $[Ca^{2+}]_i$. Agonists signaling through increased $[Ca^{2+}]_i$ (e.g., Ang II, ET-1) stimulate Nox5 *via* its Ca^{2+} binding hands (13, 33, 138, 156). Binding of calmodulin also enhances Nox5 Ca^{2+}

affinity, while Ca^{2+} /calmodulin-dependent kinase II phosphorylates Nox5 β on Ser475 to increase ROS generation (156). The ability of Nox5 to respond to Ca^{2+} -sensitive signaling may be especially important in vascular cells, where Ca^{2+} is critically involved in vascular function (contraction/dilation, growth). Of the many Ca^{2+} channels important in regulating vascular $[Ca^{2+}]_i$, transient receptor potential melastatin cation channel 2 (TRPM2) is interesting in the context of oxidative stress, as TRPM2 is highly redox sensitive (135, 203).

The significance of this novel isoform in the cardiovascular system is unknown, and to our knowledge, nothing is known about vascular Nox5 and hypertension. Since the *Nox5* gene is present in humans but absent in rodents, a study of this isoform is challenging in the experimental setting.

Antioxidant Defense Mechanisms

In biological systems, the natural defense against ROS comprises enzymatic and nonenzymatic systems. Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional factor, is the master regulator of antioxidant genes and hence of antioxidant status (44). It may be possible, although still not proved, that Nrf2 is down-regulated in hypertension, which could contribute to decreased antioxidant status and consequent oxidative stress. Nrf-2 expression and activity are impaired in conditions associated with hypertension, such as kidney disease and diabetes (101), but the role in hypertension *per se* is unknown.

Major enzymatic antioxidants, which are regulated, in part by Nrf-2, include manganese superoxide dismutase (MnSOD), catalase, glutathione peroxidases, thioredoxin, and peroxiredoxin (31, 69, 89). SOD, of which there are three isoforms, and catalyze the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2 . Of the three SOD isoforms eSOD is the main vascular SOD (69). Non-enzymatic antioxidants, which act as ROS scavengers, include vitamins A, C, and E; glutathione, bilirubin, and uric acid.

Low antioxidant bioavailability promotes cellular oxidative stress and has been implicated in cardiovascular and renal oxidative damage that is associated with hypertension. Activity of SOD, catalase, and GSH peroxidase is lower, and the GSSG/GSH is higher in plasma and circulating cells from hypertensive patients than normotensive subjects (174). Moreover, a large population-based cross-sectional study conducted in more than 20,000 adults participating in the European Prospective Investigation Into Cancer-Norfolk demonstrated that individuals with high plasma vitamin C levels had lower blood pressure than those with low plasma vitamin C levels (144).

In mice deficient in EC-SOD and in rats in which GSH synthesis is inhibited, blood pressure is elevated, indicating that reduced antioxidant capacity is associated with elevated blood pressure (42). In angiotensinogen-overexpressing mice, which are hypertensive, catalase overexpression prevented blood pressure elevation and protected against kidney damage (68). In human studies, plasma vitamin C levels are inversely related to blood pressure levels, indicating a potential blood pressure-lowering effect of this antioxidant (94).

Oxidative Stress and Experimental Hypertension

There is now extensive experimental data showing that ROS play a role in the development of hypertension, with

many models of hypertension exhibiting oxidative stress, including genetic forms (SHR, SHRSP), surgically induced (2K1C, aortic banding), hormone-induced (Ang II, ET-1, aldosterone, and DOCA), and diet-induced hypertension (salt, fat) (32, 87, 179). Oxidative stress and inflammation are common features in cardiac, vascular, renal, and retinal damage in hypertension, and especially in the context of diabetes (32) (Fig. 4). Mice deficient in ROS-generating enzymes (e.g., Nox2 - / -, p47phox - / -, and Nox1 - / -) have lower blood pressure compared with wild-type counterparts, and Ang II infusion fails to induce hypertension in these mice (15, 109). The exact mechanisms by which ROS influence blood pressure remain unclear, but many systems are involved, including the brain, heart, kidneys, and vessels. In addition, recent evidence indicates that the immune system may be involved. Oxidative stress precedes the development of hypertension in experimental models, and it may play a role in fetal programming and the development of hypertension in adult life (153). This may be related to alterations in antioxidant status, as impaired renal catalase and glutathione peroxide mRNA expression and activity were found to precede

the development of hypertension in SHR (198). Markers of oxidative stress, such as TBARS, and F₂-isoprostanes, tissue concentrations of O₂^{•-} and H₂O₂, and activation of Nox and xanthine oxidase are increased; whereas levels of NO and antioxidant enzymes are reduced in experimental hypertension (61, 222). Moreover cross-talk between mitochondria and Noxs may amplify ROS generation (Fig. 5).

Of the many models of hypertension in which oxidative stress has been shown to be important, Ang II-dependent hypertension, is the best characterized. In Ang II-infused rats and mice, expression of Noxs (Nox1, Nox2, and Nox4), Nox activity, and ROS generation are increased (27, 226). Ang II-induced hypertension is also associated with DNA double-strand breaks and the mutagenic DNA base modification 7,8-dihydro-8-oxo-guanine, effects that were blocked by the radical scavenger tempol (21). These data demonstrated oxidative stress-mediated genotoxic effects of Ang II *in vivo*, which may contribute to oxidative cardiovascular and kidney damage in hypertension (129). In p47phox knockout mice and in gp91phox (Nox2) knockout mice, Ang II infusion failed to induce hypertension, and these animals do not show the same

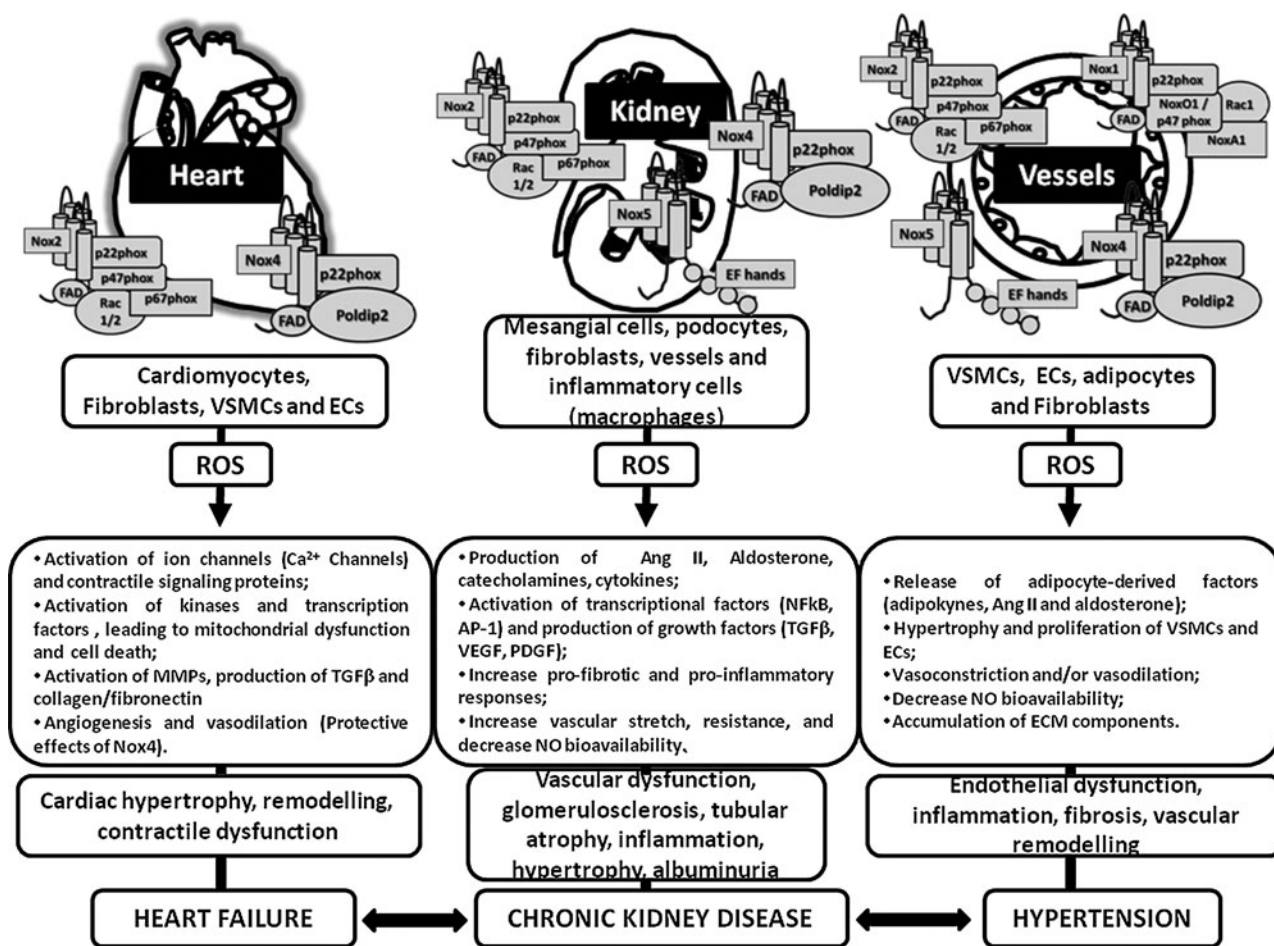


FIG. 4. Nox distribution in the heart, kidney, and vessels. Nox homologues are differentially expressed in tissues from the cardiovascular-renal systems. As illustrated in the figure, Noxs not only play important roles in pathological conditions, but also regulate a series of responses that are important to the physiology of each cell type or tissue. An increase in ROS generation and a dysregulation of Nox expression/activity, followed by a decrease in ROS degradation, leads to an increase in ROS-induced injurious actions and oxidative damage. MMP, matrix metalloprotease; VSMC, vascular smooth muscle cells; EC, endothelial cells; ECM, extracellular matrix.

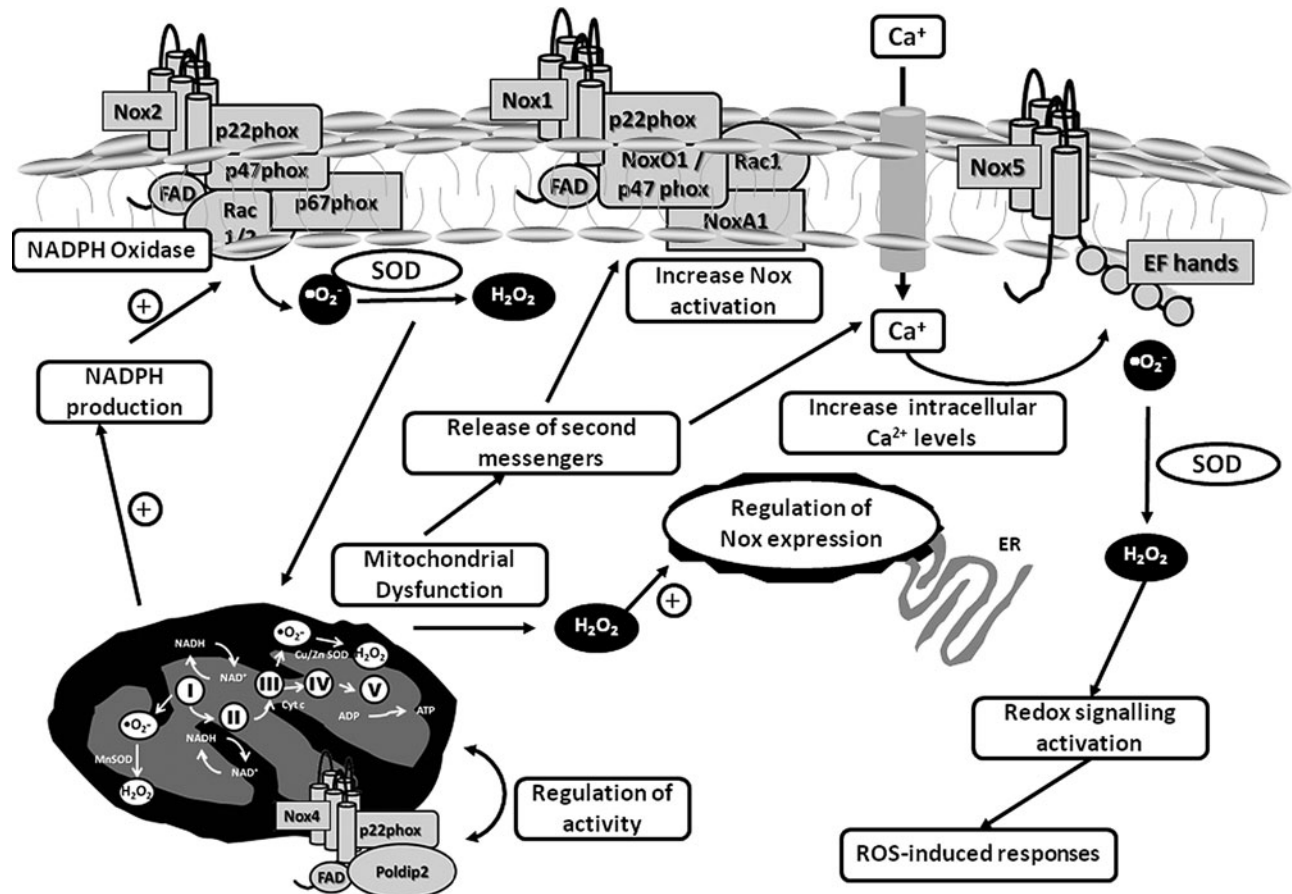


FIG. 5. Interactions between Nox and mitochondria. Noxs can regulate mitochondrial function and vice versa. Through production of ROS, Noxs are able to induce mitochondrial dysfunction, leading to an increase in the production of ROS by the mitochondria. Either by ROS generation or by release of second messengers, the mitochondria will increase the expression and activation of Noxs, further increasing oxidative stress, activation of redox signaling, and ROS-induced effects. It has also been reported that Noxs and mitochondria colocalize and may regulate their respective activities.

augmentation in $O_2^{\bullet-}$ production, vascular hypertrophy, and endothelial dysfunction observed in wild-type counterparts (110). In Ang II-infused mice treated with siRNA targeted to renal p22phox, renal Nox activity was blunted; ROS formation was reduced; and blood pressure elevation was prevented (136). On the other hand, overexpression of vascular p22phox was associated with increased oxidative stress and vascular dysfunction, but no significant increase in blood pressure (114). Treatment with non-specific pharmacological inhibitors of Nox, such as apocynin or diphenylene iodonium, or gp91dstat, a novel-specific inhibitor of Nox, reduced vascular $O_2^{\bullet-}$ production, prevented cardiovascular remodeling, and attenuated development of hypertension in Ang II-dependent hypertension (73, 166). Nox1-deficient mice have reduced vascular $O_2^{\bullet-}$ production, and blood pressure elevation in response to Ang II is blunted (63); whereas in transgenic mice in which Nox1 is overexpressed in vascular smooth muscle cells in the vascular wall, Ang II-mediated vascular hypertrophy and blood pressure elevation are enhanced (49). However, in a model of chronic Ang II-dependent hypertension, where transgenic mice expressing human renin (which exhibit an Ang II-sensitive hypertensive phenotype) were crossed with Nox2 $^{-/-}$ or Nox1 $^{-/-}$ mice, development of hypertension was not prevented even though

oxidative stress was reduced, suggesting that Noxs may be more important in acute than in chronic hypertension (234). It should be stressed that in these Nox knockout or transgenic studies, baseline cardiovascular phenotypes of mice are surprisingly normal and it is only in the context of a challenge, such as with Ang II or salt, that mice exhibit vascular and blood pressure aberrations.

Oxidative stress in the brain plays an important role in the development of hypertension (103, 231, 239). Nox-induced ROS production in the rostral ventrolateral medulla causes sympathoexcitation in hypertensive rats, through mechanisms that involve NO and pro-inflammatory processes. Transgenic (mRen2)27 rats have increased medullary tissue Nox activity and increased ROS production in isolated mitochondria (146). Free radical signaling in the subfornical organ (SFO), an important forebrain circumventricular organ, is critical for sympathetic activation, driving the elevation in blood pressure in Ang II-infused mice (29). Suppression of ROS generation in the SFO by overexpression of CuZn-SOD prevented development of hypertension in these mice. The SFO mediates ROS-related effects through activation of the paraventricular hypothalamic nucleus, causing increased plasma vasopressin, up-regulation of endothelin-1 in cerebral resistance arterioles, and activation of endothelin type A

receptors and through activation of cerebrovascular AT₁ receptors by Ang II (29). Both pathways mediate vasomotor dysfunction by inducing vascular oxidative stress. The findings implicate the SFO and its efferent hypothalamic pathways in the cerebrovascular alterations induced by Ang II, and they identify vasopressin and endothelin-1 as potential therapeutic targets to counteract the damaging effects of hypertension on the brain.

Although it is becoming increasingly clear that ROS produced in the central nervous system promote sympathetic outflow, inflammation, and hypertension, the contribution of Noxs to these processes is unclear (38). In mice in which p22phox was deleted in the SFO, Ang II infusion failed to elicit a hypertensive response (120). These findings confirmed the importance of Noxs in the SFO as a critical determinant of the blood pressure and vascular inflammatory responses to Ang II (120). In addition to Nox-derived ROS, ER stress in the brain SFO is important in Ang II-induced hypertension (235).

Additional sources of Nox-generated ROS in Ang II-induced hypertension are cells of the immune system, specifically T cells. In mice which lack lymphocytes (RAG1^{-/-} mice), hypertensive responses to Ang II are reduced, a response that is restored by adoptive transfer of T-lymphocytes, but not of B-lymphocytes (223). T cells influence blood pressure elevation by interacting with B7 ligands (CD80 and CD86) and the T-cell coreceptor CD28 (223) and through the dysregulation of T-regulatory and T-effector cells (14). Adoptive transfer of T-regulatory lymphocytes cells suppresses Ang II-mediated vascular injury and hypertension, in part, by reducing Nox-derived ROS generation (14).

Other atypical sources of ROS that may impact vascular redox status include perivascular adipose tissue (23, 80, 148). Adipose tissues possesses functionally active Nox4, which generates ROS, an important modulator of adipocyte biology and adipocytokine production. Perivascular adipose tissue influences vascular tone through adipocyte-derived vasoactive factors and ROS, effects that may be enhanced in hypertension, especially in the context of obesity, metabolic syndrome, and diabetes (148).

In order to further support a role for oxidative stress in experimental hypertension, treatment of hypertensive rats or mice with antioxidant vitamins, SOD mimetics (tempol [4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl]), free radical scavengers, or tetrahydrobiopterin (BH4) attenuate or prevent development of hypertension and its associated target organ damage (37, 72).

ROS and Noxs in Human Vessels

Although vascular Noxs have been well characterized in experimental models of hypertension, with most studies demonstrating increased expression and activity of Nox1, Nox2, and Nox4 in a site- and cell-specific manner, little is known about vascular Noxs in human hypertension. Characterization of Noxs in human vessels has focused mainly on discarded surgical tissue from patients undergoing bypass surgery. Results from extensive human studies by the Channon group showed increased vascular Nox activity and expression of Nox2, Nox4, and p22phox, but not of Nox1, in patients with coronary artery disease or diabetes (10, 76–78). Others showed that vascular Nox5 expression is increased in patients with atherosclerosis and cardiac disease (18, 178,

195). Hahn *et al.* demonstrated the presence of Nox5 expression in human intramyocardial blood vessels and cardiomyocytes, with significant increases after myocardial infarction (79). In a recent comprehensive descriptive study, Pandey *et al.* (157) reported that Nox5 is present in human saphenous vein and internal mammary artery, cultured human VSMCs, and endothelium, but not in fibroblasts, with the α and β isoforms being most abundant in vascular cells. Adenovirus-mediated overexpression of Nox5 promoted phosphorylation of MAPK in ECs and VSMCs (195), which is similar to our studies in Ang II-stimulated Nox5-containing cells (151, 228). These studies, although informative of human Nox expression, have limitations, as they are essentially descriptive and do not reveal much insight into the mechanisms of oxidative stress. However, such studies consistently demonstrate up-regulation of vascular Noxs in cardiovascular disease, indicating their potential importance in such conditions.

ROS, Noxs, and Vascular Function: Clues from Studies in Patients with Hereditary Deficiency of Nox

Practical and ethical issues limit the direct study of Noxs and ROS in human cardiovascular disease. However, a series of studies by Viola and colleagues, in which patients with Nox deficiency (chronic granulomatous disease) were studied, demonstrated that Noxs and ROS are important in the regulation of vascular tone (122, 124, 225). This was evidenced by the following: patients with Nox 2 or p47phox deficiency had significantly higher forearm-mediated dilation and lower serum levels of soluble Nox2-derived peptide (marker of Nox2 activation) and 8-iso-PGF2 α levels compared with healthy subjects; platelets from patients with Nox2 deficiency have reduced isoprostane formation; patients with CGD are protected from ischemia-reperfusion injury (121, 122, 124, 225). Moreover, women carriers of hereditary deficiency of Nox2 had higher flow-mediated dilation, lower intima-media thickness, reduced urinary isoprostanes and serum Nox2 activity, increased NO bioavailability, and higher serum nitrite/nitrate compared with controls, suggesting reduced vascular damage and atherosclerotic burden in carriers of Nox2 deficiency (224). Taken together, these studies implicate a role for Nox and ROS in the regulation of endothelial function and vascular tone. However, it should be kept in mind that these findings may be influenced by the clinical conditions of these patients, as they have life-threatening infections, that are often treated prophylactically with antibiotics and antifungal agents.

ROS, Oxidative Stress and Human Hypertension

Hypertensive patients exhibit higher levels of plasma H₂O₂, increased plasma, and urine markers of oxidative stress such as TBARS, oxidized low-density lipoprotein (oxLDL), and 8 iso-prostane and reduced antioxidant capacity, compared with normotensive subjects (20, 171, 184, 205, 229). Isoprostanes are stereoisomers of prostaglandins, which are formed mainly by non-enzymatic peroxidation of arachidonic acid by ROS. Plasma levels of oxLDL are influenced primarily by the magnitude of oxidative stress within the vascular wall as well as by the susceptibility of LDL to oxidation. Treatment with antihypertensive drugs reduces oxidative stress biomarkers, in some cases independently of blood pressure

lowering. In 2011, the European Food Safety Authority accepted urine isoprostanes as a biochemical marker of oxidative stress (55). However, testing of such biomarkers is not routine practice in the clinic, and standardized methodologies are not yet available at hospital laboratories.

Due to the potential predictive value of biomarkers, there is growing interest in identifying more specific and direct indices of oxidative stress. Recent advances in the field have focused on redox proteomics in which oxidative post-translational modifications can be identified in protein targets of oxidative or nitrosative stress (193). Redox proteomics technologies can identify oxidized proteins in serum, plasma, and urine. Advanced oxidation proteins are variants primarily of albumin and fibrinogen and have been identified in plasma and serum from patients with chronic kidney disease and hypertension (173, 183). Post-translational modifications of protein residues by ROS include thiol oxidation and carbonylation. In a large cohort of Chinese adults, plasma reactive carbonyl species was positively associated with blood pressure levels and was found to be an important risk factor for developing hypertension (36). Nitration of proteins, especially modification of tyrosine to 3-nitrotyrosine, is another form of redox modification and is increased in serum proteins in patients with hypertension, chronic kidney disease, and diabetes (25, 131, 217).

Normotensive subjects with a family history of hypertension have greater ROS production than blood pressure-matched subjects without a family history of hypertension, suggesting a genetic component that is associated with elevated production of free radicals (57, 105, 108). Racial differences in oxidative stress and inflammation have also been demonstrated. Human umbilical vein endothelial cells (HUVEC) from African Americans exhibited higher levels of NO, IL-6, p47phox, Nox2, and Nox4 and lower SOD activity than HUVECs from Caucasians (169).

ROS production is increased in VSMCs from resistance arteries of hypertensive patients, and this is associated with up-regulation of vascular Nox (75, 112, 142, 207, 213, 233). The importance of Nox in human cardiovascular disease is supported by studies showing that polymorphisms in Nox subunits are associated with increased atherosclerosis and hypertension. In particular, the -930(A/G) polymorphism in the p22(phox) promoter may be a novel genetic marker that is associated with hypertension. An association between the p22phox -930 G polymorphism has been associated with blood pressure in normotensive subjects (100). The C242T CYBA polymorphism is associated with essential hypertension, and hypertensive patients carrying the CC genotype of this polymorphism exhibit features of Nox-mediated oxidative stress and endothelial damage and are prone to cerebrovascular disease. The T allele of the p22-phox C242T polymorphism is also associated with higher left ventricular mass/height and increased Nox activity in Brazilian hypertensive patients, suggesting that genetic variation within Nox components may modulate left ventricular remodeling in subjects with hypertension (176). In a Japanese population, the G(-930)A polymorphism of CYBA was confirmed to be important in the pathogenesis of hypertension (141). Polymorphisms of the xanthine oxidase gene (-337GA and 565+64CT) have also been shown to be related to blood pressure and oxidative stress in hypertension (237).

In addition to essential hypertension, oxidative stress is found in other forms of hypertension. Patients with primary hyperaldosteronism exhibit increased levels of plasma ROS and markers of subclinical inflammation compared with essential hypertensive patients (60, 196). These findings were associated with increased cardiac fibrosis, phenomena that were independent of blood pressure elevation but related to proinflammatory and oxidative stress effects of aldosterone. Hypertension during pregnancy was also found to be associated with oxidative stress as evidenced by increased TBARS levels during labor (5). This was related to reduced plasma SOD activity and increased plasma GSH-Px with no change in GSH-Red activity (5). Alterations in antioxidant and pro-oxidant status during pregnancy may constitute an increased risk factor for hypertensive pregnant women. Elderly patients (~75 years) who exhibit endothelial dysfunction and decreased antioxidant capacity responded positively to oral antioxidant therapy, with improved flow-mediated vasodilation, decreased plasma TBARS levels, and increased antioxidants (230). However, in young individuals (~25 years), antioxidant therapy worsened endothelial function, suggesting that the age-related impairment is attributed, at least in part, to oxidative stress.

Decreased antioxidant defense mechanisms also contribute to oxidative stress in human hypertension. Patients with essential hypertension exhibit reduced activity and decreased plasma levels of antioxidant enzymes, including SOD, glutathione peroxidase, and catalase (192). Decreased levels of antioxidant vitamins A, C, and E have been shown in newly diagnosed, untreated hypertensive patients compared with normotensive controls (143). Nrf-2, the master transcription factor that regulates antioxidant genes, is protective in maternal diabetes-induced perinatal hypertension (30, 221). Antioxidant vitamins reduced blood pressure and arterial stiffness in patients with diabetes (240), but had no effect in postmenopausal women or in healthy subjects (140). Population studies have demonstrated an inverse association between plasma vitamin C levels and vitamin C consumption with blood pressure (28, 139), and a recent meta-analysis reported that vitamin C supplementation reduced systolic and diastolic blood pressure (147). In patients with white coat hypertension, serum protein carbonyl (indicating protein oxidation) was increased and endogenous antioxidant proteins (protein thiol, SOD, glutathione) were decreased compared with normotensive individuals, further supporting a relationship between low antioxidant capacity, increased oxidative stress, and hypertension (201).

ROS as Therapeutic Targets in Human Hypertension

Considering the possible pathophysiological role of oxidative stress in hypertension and other cardiovascular disorders, and the convincing experimental data, it is reasonable to imagine that reducing ROS bioavailability through antioxidants, ROS scavengers, and Nox inhibitors would have protective and blood pressure-lowering effects. Antioxidants that have been commonly studied, including vitamins A, C, and E, co-enzyme Q, beta carotene, polyphenols, and flavonoids. A recent meta-analysis evaluating the effects of vitamin C supplementation on blood pressure reported that vitamin C supplementation reduces blood pressure by 3.84/1.48 mm Hg (147). However, findings are inconsistent, and clinical trial

data are inconclusive, with most large antioxidant clinical trials failing to demonstrate beneficial cardiovascular effects (129, 133, 187). Findings from the Physicians Health Study II, a randomized double-blind, placebo-controlled trial of a common daily multivitamin in which 14,641 men were studied for 11.2 years, revealed no significant effect of multivitamin supplementation on cardiovascular events (188). Similar negative results have been reported for many other large antioxidant trials, which have been recently reviewed (83, 85, 97, 129, 133, 187). In addition, clinical trials examining the effects of antioxidant vitamins (vitamins C and E) in the prevention of pre-eclampsia and gestational hypertension have been negative (132, 168). Reasons for these disappointing results are numerous, but as Brieger *et al.* (22) hypothesize, "antioxidant supplementation is too late, too little and too non-specific."

Based on the lack of evidence proving antioxidant benefits in cardiovascular diseases, antioxidant supplementation is not recommended for the prevention or treatment of hypertension. However, most hypertension guidelines recommend that the general population consumes a diet rich in fruits, vegetables, and whole grains, which is a diet rich in antioxidants (123). The low sodium Dietary Approaches to Stop Hypertension (DASH) diet reduces oxidative stress and improves vascular function in salt-sensitive patients (6, 88).

Another important lifestyle modification that may have cardiovascular protective and blood pressure-lowering effects by reducing oxidative stress is exercise. In experimental models of hypertension and in patients with coronary artery disease, exercise reduced vascular Nox activity and ROS production, ameliorated vascular injury, and reduced blood pressure (1). Resistance training in men decreased circulating levels of matrix metalloproteinase-9 and 8-isoprostane (43). However, in elderly patients, combining antioxidant therapy with exercise negated blood pressure-lowering beneficial effects of exercise (43).

Clinical studies examining the effects of xanthine oxidase inhibitors (70, 204), tetrahydrobiopterin (sapropterin dihydrochloride [6r-bh4]) (167), and N-acetylcysteine (165) have demonstrated improved vascular function and blood pressure lowering in patients with hypertension, chronic kidney disease, and pulmonary hypertension. However, a recent clinical trial demonstrated that in patients with CAD, oral tetrahydrobiopterin treatment failed to improve endothelial function or cardiovascular outcomes, possibly due to auto-oxidation of the compound (47).

Some of the beneficial effects of classical antihypertensive agents such as β -adrenergic blockers, ACE inhibitors, AT₁ receptor antagonists, and Ca²⁺ channel blockers may be mediated, in part, by decreasing vascular oxidative stress (59, 125, 154). These effects have been attributed to the direct inhibition of Nox activity and to intrinsic antioxidant properties of the drugs. However, some studies failed to show changes in oxidative stress despite significant blood pressure lowering by classical antihypertensive drugs (106, 191).

Other commonly used drugs have also been shown to reduce oxidative stress in patients with cardiovascular risk factors. Fenofibrate, a lipid-lowering agent with pleiotropic actions, improved endothelial function, measured by brachial flow-mediated dilation, in middle-aged and older normolipidemic adults by reducing oxidative stress and by increasing endothelial NOS expression and activity (227).

Nox as Putative Targets in the Treatment of Hypertension

Antioxidants and radical scavengers increase rates of ROS degradation, whereas inhibitors of ROS-generating enzymes decrease rates of ROS formation. Of the many enzymes that are potential therapeutic targets are Noxs. Due to this, there has been enormous interest in the development of agents that inhibit Noxs in an isoform-specific manner (91, 102, 194, 197). Different strategies have been employed, including small-molecule inhibitors, peptide Nox inhibitors, and siRNAs. Several pharmacological compounds have been registered as Nox inhibitors in the patent literature (102). First-generation Nox inhibitors, including apocynin and diphenylene iodonium, are non specific, lack selectivity, and have multiple "off-target" side effects. Newer-generation NOX inhibitors are more specific and selective. To date, two different classes of compounds have been claimed as potent selective and orally active bioavailable Nox inhibitors: pyrazolopyridines (GKT136901 and GKT137831) and triazolopyrimidine derivatives (VAS2870 and VAS3947) (39, 91, 102, 194, 197). These agents target mainly Nox1 and Nox4, and, apparently, have a few "off-target" side effects (22, 217). The exact mechanisms by which GKT compounds inhibit Nox activity remain unclear, but they may act as competitive substrate inhibitors, as they structurally resemble NADPH. GKT137831 has already undergone safety studies in humans and was found to be safe and well tolerated (86). Its use will soon be tested in patients with diabetic nephropathy, and future studies may evaluate the effects on hypertension and other cardiovascular diseases (86). Although much research is still needed in the field, the clinical utility of Nox-specific inhibitors is promising.

Conclusions

Compelling findings from experimental and animal studies indicate a role for oxidative stress and Noxs in the etiology of hypertension. However, there is no direct clinical proof that oxidative stress causes hypertension in humans. What is clear from experimental and translational research is that dysregulation of Noxs increased ROS generation-altered redox signaling, and oxidative injury may be important in the pathophysiology of increased blood pressure, in large part through effects on endothelial function, vascular tone, arterial remodeling, and vascular inflammation. The recently identified Nox, Nox5, may be particularly important in vascular injury and cardiovascular disease in humans. More translational and clinical research in the field of oxidative stress and hypertension is needed, especially in the development of sensitive, specific, and reliable biomarkers and assays to assess the redox status of humans in health and disease. Clinical trials are designed to address the role of ROS specifically in the development of hypertension. With a better understanding of ROS (patho)biology in humans, it should be possible to target therapies more effectively so that damaging effects of ROS can be prevented or ameliorated. Such approaches may have potential benefit in the treatment of redox-sensitive pathologies that are associated with cardiovascular disease, including hypertension.

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Address correspondence to:

Dr. Rhian M. Touyz
 Institute of Cardiovascular and Medical Sciences
 BHF Glasgow Cardiovascular Research Centre
 University of Glasgow
 126 University Place
 Glasgow G12 8TA
 United Kingdom

E-mail: rhian.touyz@glasgow.ac.uk
 rtouyz@uottawa.ca

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Abbreviations Used

Ang II = angiotensin II
 BH4 = tetrahydrobiopterin
 CKD = chronic kidney disease
 ECM = extracellular matrix
 ECs = endothelial cells
 eNOS = endothelial nitric oxide synthase
 H₂O₂ = hydrogen peroxide
 HUVEC = human umbilical vein endothelial cells
 MAPK = mitogen-activated protein kinases
 MMP = matrix metalloprotease-9
 MnSOD = manganese superoxide dismutase
 NADPH = nicotinamide adenine dinucleotide phosphate
 NO = nitric oxide
 NOS = nitric oxide synthase
 Noxs = NADPH oxidase
 Nrf2 = nuclear factor erythroid 2-related factor 2
 oxLDL = oxidized low-density lipoprotein
 PDGF = platelet-derived growth factor
 ROS = reactive oxygen species
 SFO = subforminal organ
 TBARS = thiobarbituric acid-reactive substances
 TRPM2 = transient receptor potential melastatin cation channel 2
 TSP1 = thrombospondin-1
 VSMC = vascular smooth muscle cells