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Forum Review

NAD(P)H Oxidase-Derived Reactive Oxygen Species as Mediators of Angiotensin II Signaling

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ABSTRACT

Angiotensin II has been shown to participate in both physiological processes, such as sodium and water homeostasis and vascular contraction, and pathophysiological processes, including atherosclerosis and hypertension. The effects of this molecule on vascular tissue are mediated at least in part by the modification of the redox milieu of its target cells. Angiotensin II has been shown to activate the vascular NAD(P)H oxidase(s) resulting in the production of reactive oxygen species, namely superoxide and hydrogen peroxide. In this article, we review what is known about the molecular steps that link angiotensin II and its receptor to production of reactive oxygen species and subsequent redox-mediated events, focusing on the structural and functional properties of the vascular NAD(P)H oxidases and their downstream mediators. As such, we provide a framework linking angiotensin II to crucial vascular pathologies, such as hypertension, atherosclerosis, and restenosis after angioplasty, by means of the NAD(P)H-dependent oxidases and their effector molecules. *Antioxid. Redox Signal.* 4, 899–914.

INTRODUCTION

THE RENIN–ANGIOTENSIN SYSTEM (RAS) is an important regulator of blood pressure and fluid and electrolyte homeostasis. Angiotensin II (Ang II), the main effector of the RAS, exerts its effects on multiple target organs, including the adrenal cortex, myocardium, vascular smooth muscle, endothelium, kidney, and brain, leading to the modulation of aldosterone release, heart rate and contractility, vascular tone, glomerular filtration and sodium reabsorption, and pituitary secretion of vasopressin. In addition, Ang II plays a central role in the progression of diseases in its target organs, and has been implicated in atherosclerosis (151, 159), hypertension (66), left ventricular dysfunction (94), glomerulosclerosis (32, 69), nephrosclerosis (63), and renal interstitial fibrosis (155). Recently, it has become clear that many of these effects of Ang II are mediated by reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). ROS are produced in virtually all cardiovascular and renal tissues, including the intima (80), media (78, 103), and adventitia (91) of blood vessels, heart (82), and the kidney (70). The mechanisms by which ROS are produced in these cells and modulate the function of each of the Ang II-responsive cell types that comprise these tissues will be discussed in this review, with particular emphasis on the vascular system.

NONPHAGOCYTIC NAD(P)H OXIDASES

Various investigators identified endothelial-associated xanthine oxidase (95, 105, 162), cyclooxygenase (54, 64, 137), and cytochrome P450 (31) as prime sources of vascular $O_2^{\cdot -}$. However, recent work has shown that NAD(P)H oxidoreductase systems expressed in smooth muscle, endothelial, and adventitial cells are predominant generators of ROS in the vessel wall (79, 80, 90, 103). $O_2^{\cdot -}$ production catalyzed by these oxidase systems results from the transfer of a single electron from NADH or NADPH to molecular oxygen according to the reaction:

$$NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + H^+ + O_2^{\cdot -}$$

The vascular oxidases share interesting functional homology with the well studied phagocyte respiratory burst oxidase,

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which uses a similar enzymatic pathway in its generation of ROS. This observation has led several investigators to postulate that phagocyte-like NAD(P)H oxidases function in the blood vessel wall.

THE STRUCTURE OF THE NAD(P)H OXIDASES

The neutrophil enzyme

The neutrophil NAD(P)H oxidase is composed of six subunits, four of which were initially identified through studies with cells from patients with chronic granulomatous disease (Fig. 1) (71). The two transmembrane proteins p22phox and gp91phox associate in a one-to-one stoichiometry to form cytochrome b558 (147), characterized by an unusually low midpoint potential at −245 mV. The four cytosolic components p47phox, p67phox, p40phox, and the small-molecular-weight G protein rac translocate to the membrane and assemble with the cytochrome b558 upon activation of the oxidase. Another low-molecular-weight G protein, Rap1A, copurifies with the membrane-bound subunits; however, its exact role in the enzyme complex remains unclear (71).

The β-chain of the cytochrome b558, or gp91phox, comprises the electron transfer activity of the enzyme. This subunit is a 91-kDa glycosylated protein carrying the functional groups that allow the movement of electrons from NADPH to molecular oxygen. The carboxy terminus contains both NADPH and FAD binding sites, whereas the amino terminus holds two nonidentical heme groups sandwiched between the transmembrane α-helices. These electron transfer groups are organized in order of increasing redox potentials, thus providing an energetically favorable sequence for the movement of electrons (71).

The α-chain of the cytochrome, or p22phox, has regulatory and stabilization functions. In neutrophils, this 22-kDa protein is required for the maturation and membrane expression of gp91phox (25, 158). Phagocytes lacking p22phox do not express cytochrome b, and are thus unable to produce the burst in $O_2^{\cdot -}$ needed for microbial killing (71). Moreover, p22phox may act as a linker between gp91phox and the cytosolic subunits of the oxidase. In support of this concept, Dahan *et al.* (23) identified three stretches of amino acids, in addition to the known proline-rich region, that bind both p47phox and p67phox. Further insight into the regulatory role of p22phox was derived from a series of experiments performed in intact neutrophils, demonstrating that several neutrophil agonists induce p22phox phosphorylation in a time course comparable to the activation of the phagocyte burst oxidase, suggesting that phosphorylation of p22phox may impact oxidase activation (106).

p47phox, p67phox, and rac are essential components of the neutrophil oxidase in the intact cell. Upon exposure of phagocytes to a variety of biological or chemical stimuli, these proteins assemble with the cytochrome b558, resulting in a conformational change that activates and stabilizes the enzyme. Mutations affecting any of these three proteins impair the microbicidal properties of phagocytes (5). The role of p40phox in the multicomponent enzyme has not been clearly established. The available data suggest that p40phox may increase the affinity of p47phox for cytochrome b, thus facilitating the assembly and potentiating the activity of the enzyme. This protein, however, does not seem to be indispensable for oxidase activity, based on *in vitro* reconstitution of the enzyme (22).

The vascular enzymes

The components of the vascular NAD(P)H oxidases are less well characterized, and differ from one cell type to the other (Fig. 1). By using a variety of techniques, gp91phox, p22phox, p47phox, and p67phox have been identified in endothelial cells (11, 43, 61) and adventitial fibroblasts (91). On the other hand, vascular smooth muscle cells (VSMCs) were found to express p22phox (36), p47phox (93), and rac, but relatively little gp91phox (67). Recent data suggest that

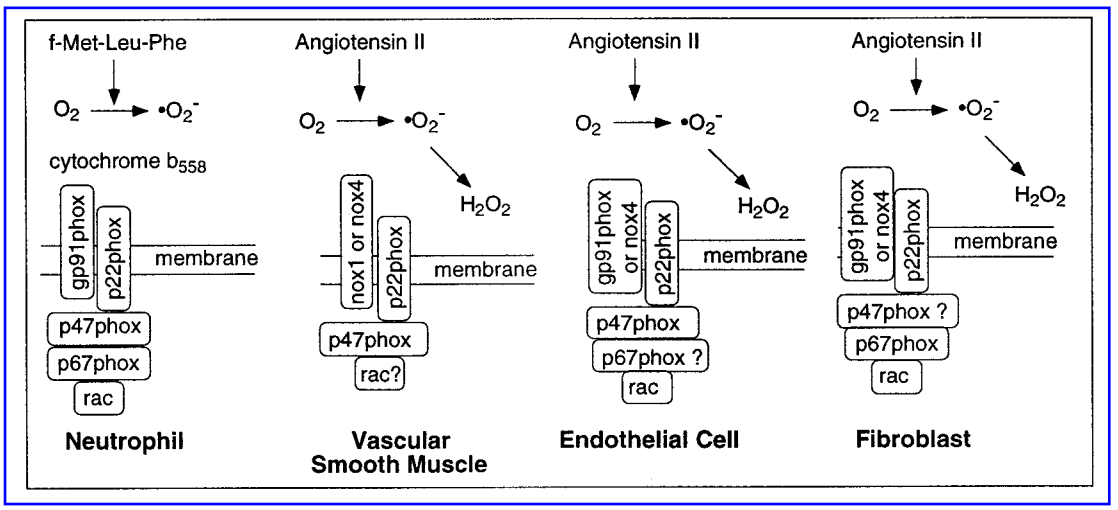

FIG. 1. NAD(P)H oxidases in vascular cells. The structure of the NAD(P)H oxidase differs among cell types. The molecular structures depicted here for each cell type are derived from a compilation of published literature.

certain types of VSMCs may in fact express detectable gp91phox, depending on their origin (2, 138).

The structural heterogeneity of the vascular NAD(P)H oxidase is further compounded by the kinetic dissimilarity between the vascular enzymes and their phagocytic counterpart. Whereas the neutrophil oxidase is inactive under unstimulated conditions, a baseline production of ROS can be detected in the vessel wall. Upon stimulation, the vascular flavin oxidase produces a low level of ROS that is about one-third that of stimulated neutrophils, but the response is more sustained when compared with the burst of free radical output by phagocytic cells (46). Ang II has been shown, both *in vitro* and *in vivo,* to be one of the most potent stimulants of the vascular NAD(P)H oxidases (47, 92, 161), with ROS acting as signaling molecules for downstream effectors involved in a variety of pathogenic processes (see below).

In VSMCs, the role of the phagocytic subunits in NAD(P)H-dependent $O_2^{\cdot-}$ production has been established in a variety of experimental designs using antisense, overexpression, antibody interference, and gene knock-out techniques. The small cytochrome b558 subunit, p22phox, is the component that has been most extensively studied in Ang II-responsive tissues. Stable transfection of antisense p22phox results in a 50% decrease in $O_2^{\cdot-}$ production in Ang II-stimulated cells as compared with controls (142). Furthermore, rats made hypertensive by infusion of Ang II show an increase in p22phox mRNA, with a parallel enhancement in NAD(P)H oxidase activity when compared with sham-operated animals, suggesting that Ang II could increase vascular NAD(P)H oxidase function in part through the modulation of p22phox expression (37). Busse's group extended these results to VSMCs exposed to stimuli other than Ang II. They showed that platelet-derived growth factor (PDGF) induces a time- and concentration-dependent increase in ROS production that is associated with a parallel increase in p22phox expression, and is inhibited by diphenylene iodonium (DPI), an inhibitor of flavin-containing oxidases, and by antisense p22phox (42). The central role of p22phox in the formation of a functional NAD(P)H oxidase is not restricted to VSMCs. Using cardiac microvascular endothelial cells, Xie *et al.* (156) showed that Ang II-induced activation of the NAD(P)H oxidase is accompanied by an increase in p22phox mRNA.

Using murine VSMCs derived from the explanted aortas of wild-type and p47phox knock-out mice, Lavigne *et al.* (68) showed a significant decrease in phorbol myristate acetate (PMA)- or Ang II-stimulated $O_2^{\cdot-}$ production in cells from animals lacking p47phox, but not in those from wild-type animals. This decrease in response to agonist stimulation was restored after transduction of the cells with p47phox. Further evidence for the participation of p47phox in vascular $O_2^{\cdot-}$ production comes from a recent study conducted by Barry-Lane *et al.* (10). Their results showed that aortic VSMCs from mice lacking the p47phox gene have a decreased ability to produce ROS in response to agonist stimulation as compared with aortas from wild-type animals.

Although a role of rac in activation of the nonphagocytic oxidase has been clearly established, and, in the absence of p67phox, its binding partner has not been clearly identified. Results obtained from experiments with dominant-negative rac in cultured fibroblasts point toward the necessity of a functional GTPase for oxidase activity (60). A similar role for rac-1 in NAD(P)H-dependent ROS generation was established in endothelial cells exposed to mechanical shear stress. The expression of a dominant-negative rac-1 gene product in bovine aortic endothelial cells blunted the increase in ROS production in response to mechanical stimulation (157). Rac-1 has also been implicated in Ang II-responsive $O_2^{\cdot-}$ production in VSMCs (121).

In contrast to VSMCs, adventitial fibroblasts and endothelial cells do express p67phox (61, 92). The amino acid sequence of the fibroblast p67phox shares an 89% identity with its phagocytic counterpart. Ang II induces an increase in p67phox mRNA expression that temporally precedes the increase in oxidase activity, and immunodepletion of p67phox results in the loss of NAD(P)H-dependent $O_2^{\cdot-}$ production, a function restored following the reintroduction of recombinant p67phox (92). These results suggest that there is both a structural and functional homology between the fibroblast and neutrophil p67phox proteins.

Whereas the vascular media of large arteries lacks gp91phox (67, 129), this protein has been identified in the intima and adventitia, where it catalyzes the generation of $O_2^{\cdot-}$ in the presence of NAD(P)H. Görlach *et al.* (43) were able to detect gp91phox and p22phox at both the mRNA and protein levels in cultured human and bovine endothelial cells, and further showed an endothelium-dependent decrease in the NAD(P)H-dependent, PMA-induced production of ROS in aortas of gp91phox knock-out mice as compared with control animals. The structure and localization of these two transmembrane components of the oxidase in coronary microvascular endothelial cells were studied by Bayraktutan *et al.* (12). Their results showed a high degree of homology in the amino acid sequences of the α-subunit in endothelial cells, VSMCs, and phagocytic cells derived from a variety of species, including rodents and humans. A similar result was obtained for the sequence of gp91phox. The endothelial protein retained the amino acid stretches necessary for the preservation of function, including the sites involved in electron transport, except for an amino acid substitution at the NAD(P)H binding site that the authors argued could affect the substrate preference of the enzyme (12). The fibroblast NAD(P)H oxidase was examined by Pagano *et al.* (91). Using immunohistochemical techniques, they demonstrated the presence of gp91phox in adventitial fibroblasts of rabbit aortas. This protein colocalizes with the sites of increased ROS production upon Ang II stimulation.

One of the recent exciting developments in our understanding of the structure–function characteristics of the vascular NAD(P)H oxidases came from the discovery of the nox family of proteins. Based on knowledge derived from the study of the phagocyte enzyme, researchers screened cDNA libraries in an attempt to identify a protein homologous to the neutrophil gp91phox that would act as a catalytic subunit in nonphagocytic oxidases (7, 131). Nox-1, a 564-amino acid, 65-kDa protein that shares 56% sequence homology with gp91phox, was identified. More detailed examination of its composition revealed a hydrophobicity profile similar to that of gp91phox, along with the conservation of the NAD(P)H, flavin, and heme binding sites, making it a plausible binding candidate for p22phox (7, 131). Moreover,

nox-1 expression quantified by real-time PCR in VSMCs is 2,000-fold greater than that of gp91phox, and is up-regulated in a dose-dependent manner by Ang II (67). PDGF stimulation results in a parallel increase in nox-1 mRNA levels and NAD(P)H oxidase activity (131). Finally, using antisense nox-1, Ang II-stimulated NAD(P)H-dependent production of $O_2^{\cdot -}$ by VSMCs *in vitro* is significantly blunted (67).

Another member of the nox family originally discovered in kidney (see below) has also been cloned in VSMCs (67). Nox-4 shares less homology with gp91phox than does nox-1, and dendrogram analysis clearly illustrates the distinct compositions of the two proteins. However, nox-4 still retains the putative electron transfer sites, and the hydrophobic sequences characteristic of the phagocytic cytochrome b558 β-chain (19). The regulation of nox-4 is still incompletely understood. The data currently available suggest that nox-4 mRNA is up-regulated in serum-deprived conditions, and down-regulated under growth- or Ang II-stimulated conditions (67).

Renal and cardiac NAD(P)H oxidases

Several studies, including some of the earliest investigations of neutrophil-like NAD(P)H oxidases, have tried to address the structure and function of this enzyme system in a variety of other Ang II-responsive tissues, including the heart and kidneys. Almost nothing is known about the oxidase structure in the heart, although a nonphagocytic NAD(P)H-based oxidase has been implicated in the production of $O_2^{\cdot -}$ by embryonic rat heart-derived cells (H9c2) during ischemia and subsequent reoxygenation (127). Investigation of mesangial cells, however, provided one of the first demonstrations of a functional NAD(P)H oxidase-like enzyme in nonphagocytic cells (102). Radeke *et al.* (102) used a number of approaches to characterize this enzyme system in human mesangial cells in culture. Difference spectra measurements and spectral analysis used to analyze the cytochrome were similar to those obtained from the phagocytic cytochrome b558. Furthermore, using monoclonal antibodies targeted against the α- and β-subunits of the oxidase, they confirmed the expression of these two components in their cells. Despite these similarities, the mesangial oxidase showed interesting differences in stimulus–response coupling when compared with its neutrophilic counterpart, suggesting the presence of alternative regulatory pathways in these cells. This observation was further supported by the difference in kinetics between the two enzyme systems. Whereas phagocytes are characterized by a burst of ROS production upon stimulation, adherent mesangial cells in culture showed a lower, but more sustained ROS production. Clearly, regulation of NAD(P)H oxidase activity in cells of renal origin needs to be investigated more fully.

Few studies have examined in detail the structure of the renal enzymes. Hannken *et al.* (49) showed that Ang II stimulation increased p22phox mRNA expression and $O_2^{\cdot -}$ production by renal tubular cells in culture. These changes were inhibited by the flavoprotein inhibitor DPI and by antisense p22phox oligonucleotides. Importantly, the gp91 homologue nox-4, mentioned above as being expressed in VSMCs, was originally identified in kidney cells by Geiszt *et al.* (38) and termed renox. *In situ* RNA hybridization revealed that this mRNA is highly expressed in the renal cortex, specifically in renal proximal tubular epithelial cells at sites of erythropoietin production. The oxidase function of the protein was demonstrated by increased ROS production in nox-4 transfected NIH 3T3 fibroblasts (38). Interestingly, nox-4 was simultaneously cloned by Shiose *et al.* (123). Using COS7 cells, these investigators showed that nox-4 function was independent of p22phox expression, providing another distinguishing factor between nox-4 and its homologue gp91phox. They found that nox-4 protein expression was most abundant in distal tubular renal cells, in contradiction to the result of Geiszt *et al.* (123).

Unanswered questions

Thus far, the detailed structure of the nonphagocytic NAD(P)H oxidases has not been conclusively determined, and a number of interesting questions remain to be answered. It is still unclear whether nox-1 and/or nox-4 interact with p22phox and the cytosolic phox proteins to form a functional NAD(P)H oxidase, or whether the nox proteins have p22phox-independent NAD(P)H oxidase activity. The detection of baseline unstimulated $O_2^{\cdot -}$ production by VSMCs and endothelial cells suggests the presence of a constitutively assembled NAD(P)H complex, with further modulation of activity being dependent on other regulatory steps. This regulation could be a phosphorylation reaction, the synthesis of a competing catalytic subunit with different kinetics, or other yet unidentified steps. Despite this uncertainty, it remains clear that the phox proteins play a central role in Ang II-mediated redox signaling. But, here too, the full sequence of effector molecules linking angiotensin type 1 (AT_1) receptor activation to vascular NAD(P)H oxidase stimulation remains an active area of research where much is still to be learned.

MECHANISMS OF OXIDASE ACTIVATION

As noted above, Ang II causes an NAD(P)H oxidase-dependent increase in $O_2^{\cdot -}$ production in cultured VSMCs (47), vascular endothelial cells (161), and adventitial fibroblasts (92). It has been demonstrated that the activity of the vascular NAD(P)H oxidases is regulated by Ang II on at least two levels. Long-term (hours to days) regulation of $O_2^{\cdot -}$ production is achieved via modulation of gene expression, depending on the cell type. p22phox is up-regulated in intact blood vessels in rats infused with Ang II for 5–7 days (37), nox-1 mRNA is up-regulated by Ang II in cultured VSMCs by 6–12 h (67), and p67phox levels are increased by Ang II in adventitial fibroblasts (92). Short-term (minutes) modulation of NAD(P)H oxidase activity is mediated by upstream intracellular signaling pathways and second messengers released in response to growth factor and Ang II receptor stimulation. This regulation does not depend on new mRNA synthesis, but rather likely involves assembly of the component subunits into the functional oxidase and/or biochemical modification of one of the subunits (5).

The paradigm of NAD(P)H oxidase activation in neutrophils involves the assembly of the oxidase subunits into a membrane-bound functional enzyme, as described above.

This is most likely also the case in vascular cells. Basal and Ang II-dependent $O_2^{\cdot-}$ production is inhibited in VSMCs isolated from p47phox$^{-/-}$ mice (68). Furthermore, apocynin, an NAD(P)H oxidase inhibitor that exerts its effects by blocking the translocation of the cytosolic subunits to the membrane, inhibits oxidase activity in activated endothelial cells (55).

The intracellular signaling events that link AT_1 receptor stimulation to an increase in NAD(P)H oxidase activity and ROS production are unclear at present. A role for Ca^{2+} has been suggested (29, 76). Because antioxidants do not affect either the immediate or the sustained phase of Ang II-induced Ca^{2+} mobilization in cultured VSMCs (145), it is likely that Ca^{2+} release is upstream of the NAD(P)H oxidase, although its precise role in Ang II-stimulated oxidase activation remains to be defined. An involvement of phospholipase D in NAD(P)H-driven ROS production has been documented in human VSMCs (139) and smooth muscle from human resistance arteries in hypertension (140), presumably via its ability to increase phosphatidic acid, a known activator of the neutrophil enzyme (5), and diacylglycerol, an activator of the serine/threonine protein kinase, protein kinase C (PKC). Whereas the role of this kinase in the regulation of NAD(P)H oxidase activity in nonphagocytic cells is at present controversial, neutrophil oxidase activity has clearly been shown to be regulated by PKC (29). In endothelial cells, PMA, a PKC agonist, has been shown to activate the oxidase. In VSMCs stimulated with Ang II, PKC is required for the induction of nox-1 transcription (67), but its role in the acute regulation of oxidase activity remains to be determined.

It is intriguing to speculate that, in addition to direct phosphorylation by serine/threonine-directed kinases, Ca^{2+}- and PKC-dependent tyrosine kinases may represent proximal signaling intermediates linking AT_1 receptor activation to NAD(P)H oxidase activation. Among these are focal adhesion kinase (FAK) and the proline-rich tyrosine kinase 2 (PYK2).

FAK is a 125-kDa protein that localizes to focal adhesions and is phosphorylated in response to integrin engagement (115). Interestingly, $O_2^{\cdot-}$ production by NAD(P)H oxidases is increased upon neutrophil adherence to a surface via integrins (for review, see 5). FAK phosphorylation in response to Ang II has also been well documented (45). This raises the possibility that FAK is involved in the regulation of NAD(P)H oxidase activity in response to Ang II.

By far the most intriguing tyrosine kinase is PYK2. Recent inferential evidence suggests that PYK2 might be upstream of oxidase activation. The time course of PYK2 phosphorylation by Ang II parallels closely that of NAD(P)H oxidase activation and is Ca^{2+}- and PKC-dependent (110). Both PYK2 and NAD(P)H oxidases are involved in the regulation of some of the same signaling pathways, including Akt and p38 mitogen-activated protein kinase (MAPK) (107, 143, 144). Interestingly, PYK2 and Rac-1 form a complex in response to Ang II in VSMCs (P. Rocic and P. Lucchesi, unpublished observations), suggesting a role for PYK2 in the assembly of the oxidase subunits into a functional enzyme upon Ang II stimulation. PYK2 is also an upstream regulator of FAK phosphorylation in response to Ang II (107). Another potential mechanism by which PYK2 might regulate oxidase activity is via complex formation with the epidermal growth factor receptor (EGFR) (145) followed by activation of phosphatidylinositol 3-kinase (PI3-K), similar to the mechanisms used by PDGF to stimulate $O_2^{\cdot-}$ production (6). Most importantly, PYK2 activation in response to Ang II is not redox-sensitive, as evidenced by a lack of effect of the antioxidants Tiron (an $O_2^{\cdot-}$ scavenger), *N*-acetylcysteine (NAC), and ebselen (a glutathione peroxidase mimetic) (145), although PYK2 has been shown to be responsive to H_2O_2 in VSMCs (33).

Based on this combination of published data and speculation, a picture emerges in which it is likely that NAD(P)H oxidase activation in response to Ang II depends on a large complex formation between the components of the oxidase, PKC, the EGFR, tyrosine kinases, and PYK2. It is possible that rapid activation of Ang II-dependent, redox-insensitive events, including PYK2 activation, aids in the assembly of the subunits of the oxidase required for its function.

REDOX-SENSITIVE DOWNSTREAM SIGNALING

Because Ang II activates NAD(P)H oxidases in multiple cell types, the identities of the molecular targets of ROS production are of critical importance. Many of the pathophysiological effects of Ang II are due to its growth-promoting properties: Ang II induces hypertrophy and cell cycle arrest of renal proximal tubular cells, hypertrophy of glomerular mesangial cells, VSMCs, and cardiac myocytes, and proliferation of cardiac fibroblasts. The strongest evidence for ROS mediation of Ang II signaling events revolves around those that are important for cell growth, including tyrosine kinases, MAPKs, Akt, and redox-sensitive transcription factors (Fig. 2).

Tyrosine kinases

It is well recognized that Ang II activates several tyrosine kinases that are important for its growth-promoting action. Many of these, such as c-Src, EGFR, PYK2, and janus kinase 2 (JAK2), have been shown to be activated in a redox-sensitive manner.

c-Src. c-Src is one of the kinases activated in the earliest period (<1 min) after Ang II stimulation. Activated c-Src, in turn, transduces the signal to downstream pathways: it phosphorylates phospholipase Cγ, complexes with EGFRs thereby mediating their transactivation by Ang II, and participates in the activation of MAPKs. c-Src has been shown to be stimulated by Ang II in various cell types, such as mesangial cells, cardiac fibroblasts, and VSMCs. In cardiac fibroblasts, c-Src is tyrosine-phosphorylated by Ang II and the phosphorylation is inhibited by the antioxidant NAC (148), suggesting a role for ROS in Ang II activation of c-Src. In VSMCs, Ang II rapidly induces phosphorylation of the autophosphorylation site (Y418) and the SH2 binding site (Y215) (145). Exogenously added H_2O_2 also induces the phosphorylation of these tyrosine residues, suggesting a redox sensitivity of c-Src activation. Furthermore, c-Src tyrosine phosphorylation after Ang II stimulation is inhibited by DPI, Tiron, NAC, and ebselen. These results indicate that the activation of c-Src by Ang II is mediated by NAD(P)H oxidase-derived ROS such as $O_2^{\cdot-}$ and H_2O_2.

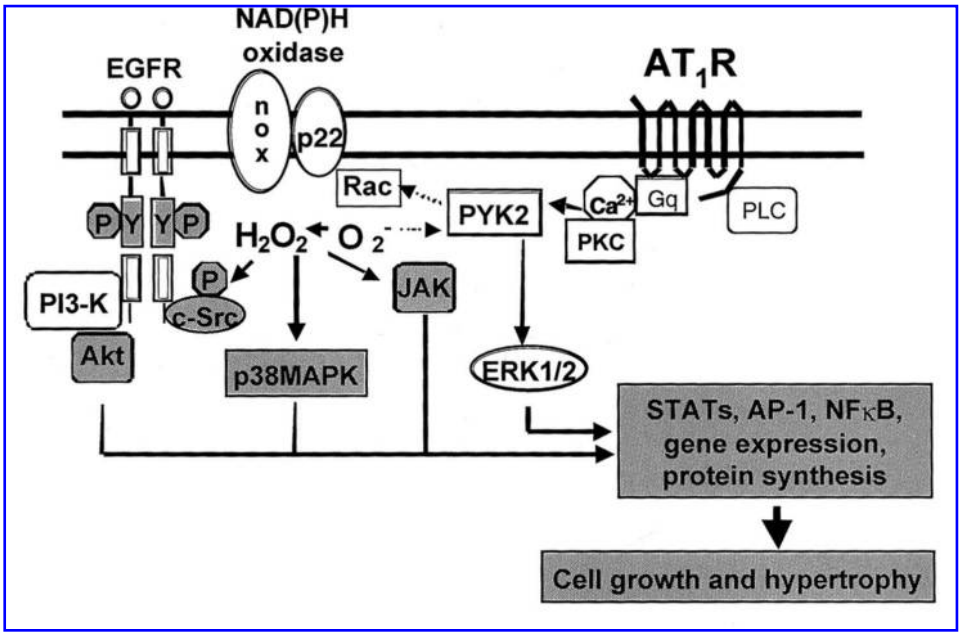

FIG. 2. Redox-sensitive signaling pathways activated by Ang II. Ang II activates both redox-sensitive (gray shading) and redox-insensitive (white shading) signaling pathways. Shown here are the signaling pathways related to Ang II-induced hypertrophy of VSMCs. Abbreviations are as indicated in the text. Gq, heterotrimeric G protein; PLC, phospholipase C; AT_1R, AT_1 receptors.

PYK2. PYK2 has been shown to be activated by Ang II in a Ca^{2+}- and PKC-dependent manner (110), making it an attractive candidate for an upstream mediator of NAD(P)H oxidase activity, as noted above. PYK2 plays an important role in protein synthesis stimulated by Ang II in VSMCs possibly by mediating the activation of extracellular signal-regulated kinase (ERK 1/2) and PI3-K (107). Some investigators have suggested that rather than being upstream of the NAD(P)H oxidase, PYK2 is in fact a target of ROS. PYK2 is tyrosine-phosphorylated after H_2O_2 stimulation in cardiac fibroblasts (148) and VSMCs (33). In one study, the phosphorylation induced by Ang II was shown to be inhibited by NAC (33); however, contrary to these results, Ushio-Fukai *et al.* (145) reported that PYK2 activation by Ang II in VSMCs was not affected by antioxidants (145). These discrepancies may be due to the phenotype of VSMCs, or the growth conditions to which they are exposed. Thus, the question of whether PYK2 is an upstream activator or a redox-sensitive downstream target of NAD(P)H oxidase-derived ROS in Ang II-responsive cells remains open.

EGFR. Growing evidence indicates that transactivation of the EGFR is an important step in the growth-promoting effect of Ang II because it is critical for the activation of MAPKs. Ang II has been shown to induce the phosphorylation of EGFRs in mesangial cells, cardiac fibroblasts, and VSMCs. In cardiac fibroblasts, EGFR phosphorylation by Ang II is inhibited by NAC (148). In VSMCs, EGFR transactivation by Ang II is also redox-sensitive, because Ang II-induced tyrosine phosphorylation of EGFR is inhibited by DPI, Tiron, NAC, and ebselen (145). This redox sensitivity was further confirmed by the fact that exogenously added H_2O_2 also induces the phosphorylation of EGFRs. Moreover, by using EGFR site-specific and phosphorylation-specific antibodies, it was demonstrated that two major autophosphorylation sites (Y1173 and Y1068) are phosphorylated after Ang II stimulation, and that phosphorylation of both sites is inhibited by NAC. Thus, ROS are considered to play a critical role in EGFR transactivation by Ang II.

JAK2. The JAK family of intracellular tyrosine kinases was originally described in cytokine signaling pathways. JAK associates with dimerized receptors after the binding of ligands such as interferon-γ, interferon-α, and interleukin-2. Intramolecular autophosphorylation then occurs, and phosphorylated JAK, in turn, activates the downstream substrates Signal Transducer and Activator of Transcription (STATs). Ang II activation of JAK2 has been reported in VSMCs (73) and rat neonatal cardiac myocytes (75). In both cell types, JAK2 directly associates with AT_1 receptors after ligand binding, and phosphorylation of JAK2 occurs. The phosphorylated JAK2 then activates STAT1 and STAT2 in VSMCs, and STAT1 and STAT3 in cardiac myocytes. Recently, an involvement of ROS in this process has been reported. Activation of JAK2 by Ang II is inhibited by DPI and electroporation of neutralizing antisera against p47phox (116). These results indicate that the $O_2^{\cdot -}$ production via the NAD(P)H oxidase is critical for JAK2 activation by Ang II.

MAPKs

MAPKs are serine/threonine kinases involved in a number of intracellular signaling pathways, including growth, apoptosis, and stress. Ang II has been shown to activate several members of MAPKs in its target cells, including ERK 1/2, c-Jun N-terminal kinase (JNK/SAPK), and p38MAPK. JNK/SAPK and p38MAPK activation by Ang II are considered to be redox-sensitive because they are inhibited by NAC

and DPI in cardiac fibroblasts (114), and by NAC and antisense p22phox in VSMCs (143, 146).

Contrary to consistent findings of redox sensitivity for JNK/SAPK and p38MAPK, the redox-sensitivity of ERK 1/2 is controversial. There have been reports demonstrating that Ang II-induced ERK 1/2 activation is inhibited by NAC and DPI in cultured renal proximal tubule cells (50) and cardiac fibroblasts (114), and by NAC in VSMCs (34). On the other hand, Ushio-Fukai *et al.* (143) and Viedt *et al.* (146) have shown that ERK 1/2 activation is not mediated by the production of ROS because it is insensitive to DPI, antisense p22phox, NAC, and overexpression of catalase. Recently, it was reported that the novel gp91phox homologue nox-1 mediates Ang II-induced $O_2^{\cdot-}$ production in VSMCs (67), thereby playing a role in redox-sensitive signaling pathways. In VSMCs infected with adenovirus to express antisense nox-1, p38MAPK, but not ERK 1/2, activation by Ang II was inhibited, confirming previous data with antioxidants. We have not yet reached a full understanding of the conditions that confer redox sensitivity to ERK 1/2.

Akt/protein kinase B (PKB)

Akt/PKB is a serine/threonine kinase that plays an important role in apoptosis regulation and protein synthesis. Akt/PKB has been shown to be activated in a redox-sensitive manner. It is stimulated by H_2O_2 and Ang II in mesangial cells (41) and VSMCs (144). Ang II-induced activation of Akt/PKB is inhibited by NAC and DPI in mesangial cells, and by DPI in VSMCs. Furthermore, Akt/PKB activation by Ang II is abolished or significantly reduced in VSMCs stably expressing human catalase (144) or in VSMCs infected with adenovirus to express antisense nox-1 (67). These results indicate that ROS production is critical for the activation of Akt/PKB by Ang II.

Transcription factors

Induction of several genes by Ang II has been shown to be redox-sensitive, including vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) (18, 141). Investigation has shown that the mechanisms responsible for this sensitivity to ROS are variable, but include the involvement of redox-sensitive transcription factors, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB).

AP-1. AP-1 is a heterodimer of the protein products of individual members of the Fos and Jun gene families. It is one of the immediate early genes and has been shown to be responsible for the hypertrophic effect of Ang II on cardiac myocytes (111). Redox sensitivity of AP-1 activation has been demonstrated by the fact that oxidative stress (H_2O_2 or ultraviolet irradiation) regulates AP-1 activity through transcriptional, posttranscriptional, and posttranslational mechanisms (57). In cardiovascular tissue, hydroperoxy fatty acid and H_2O_2 increase mRNA expression of c-Fos and c-Jun in VSMCs (104), and H_2O_2 induces complex formation between AP-1 and its target DNA in porcine aortic endothelial cells (8). In C2C12 myotubes, Ang II stimulates AP-1-derived gene transcription and AP-1 DNA binding activity (101). The effect of Ang II on the activation of AP-1 is significantly inhibited by NAC, confirming a role of ROS in Ang II-induced activation of AP-1.

NF-κB. NF-κB is perhaps the most well studied redox-sensitive transcription factor. NF-κB forms a heterodimer with inhibitor of κB (IκB) in unstimulated cells. After stimulation, IκB is degraded, allowing the translocation of NF-κB to the nucleus to activate the transcription of its responsive genes. These processes of NF-κB activation have been shown to be redox-sensitive. NAC inhibits IκB phosphorylation and degradation by tumor necrosis factor-β in human saphenous vein endothelial cells (130). H_2O_2 increases nuclear translocation of NF-κB and binding of NF-κB to its target DNA (8). In cultured endothelial cells and VSMCs, Ang II increases VCAM-1 mRNA and protein in a redox-sensitive manner (100). The Ang II-induced degradation of IκB, which leads to NF-κB activation, is inhibited by superoxide dismutase (SOD) and catalase, as well as by BTX-51702, a glutathione peroxidase mimetic that catalyzes the degradation of H_2O_2 (100). In contrast, aminotriazole, an inhibitor of catalase and glutathione peroxidase that increases intracellular H_2O_2, augments Ang II-induced degradation of IκB. These results indicate that ROS such as $O_2^{\cdot-}$ and H_2O_2 are important for the activation of NF-κB by Ang II, at least in endothelial cells.

CONSEQUENCES OF ANG II STIMULATION OF ROS

Production of ROS, as well as activation of redox-sensitive signaling pathways, has important functional consequences. Ang II-derived ROS contribute to endothelial dysfunction, VSMC growth, cardiac hypertrophy, and inflammation.

Endothelial dysfunction

One important consequence of excessive $O_2^{\cdot-}$ generation in response to Ang II stimulation is a decrease in nitric oxide (NO) bioavailability, leading to endothelial dysfunction. Endothelial dysfunction is one of the earliest manifestations of many vascular diseases, and may in fact contribute to their pathogenesis. The link between Ang II, ROS, and endothelium-dependent relaxation has been most thoroughly studied in hypertensive animals. Ang II-induced hypertension is associated with impaired relaxations to acetylcholine, the calcium ionophore A23187, and nitroglycerin (103). When the AT_1 receptor blocker losartan is administered concomitantly with Ang II, both vascular $O_2^{\cdot-}$ production and endothelium-dependent relaxations are normalized. Treatment of vessels with liposome-encapsulated SOD also enhances vasodilation in response to acetylcholine, demonstrating the key role for $O_2^{\cdot-}$ in endothelial dysfunction (13). Furthermore, genetic deletion of gp91phox improves endothelium-dependent relaxation, confirming that NAD(P)H oxidase-derived ROS are important in this process (43). In humans, increased vascular NAD(P)H oxidase activity is associated with reduced NO-mediated vasorelaxation, as well as with clinical risk factors for hypertension, restenosis, and atherosclerosis (48). Finally, administration of angiotensin converting enzyme (ACE) inhibitors to normotensive individ-

uals with coronary artery disease improves endothelial function after 6 months, suggesting that Ang II-induced $O_2^{\cdot-}$ production may chronically influence vascular tone (72).

Vascular smooth muscle growth and cardiac hypertrophy

Smooth muscle cell hypertrophy and hyperplasia contribute to the development of occlusive vascular disease and vascular remodeling in hypertension (119). Abundant *in vitro* evidence suggests that VSMC hypertrophy in response to Ang II is redox-sensitive (47, 142, 160). Ang II-induced hypertrophy can be inhibited by DPI (47), attenuation of NAD(P)H oxidase activity by transfection of antisense p22phox (142), and catalase overexpression (160). In addition, it has been shown that several redox-sensitive signaling pathways that are targets of NADPH-derived $O_2^{\cdot-}$ are required for hypertrophy, including p38MAPK and Akt (143, 144). Other vascular disorders, such as restenosis, have a significant proliferative component, resulting from smooth muscle cell and/or fibroblast migration and multiplication in the neointima (120). Growth factor-induced proliferation and migration have both been shown to require H_2O_2 (132).

In addition to their roles in VSMC growth, Ang II and ROS have also been linked to cardiac hypertrophy (82) and heart failure (59, 89). An involvement of ROS in cardiac myocyte hypertrophy *in vitro* was initially suggested by the fact that treatment with antioxidants or catalase (to scavenge H_2O_2) prevents Ang II-induced hypertrophy (82). In cardiac myocytes, merely inhibiting SOD leads to a hypertrophic phenotype, implicating $O_2^{\cdot-}$ in the hypertrophic process (124). A similar dependence upon ROS was found when norepinephrine was used to induce hypertrophy (1), suggesting that ROS may be a common signal leading to the growth response.

Inflammation

Ang II also participates in the inflammatory response in the vessel wall. It is produced by monocytes and macrophages (97) and, as noted above, induces such proinflammatory molecules as VCAM-1, MCP-1, and the thrombin receptor (16–18, 141). Induction of each of these molecules has been shown to be redox-sensitive (17, 18, 74), and for MCP-1 and the thrombin receptor, a role for ROS in Ang II-mediated gene expression has been demonstrated (17, 18). In addition, the ROS-dependent, thrombin-induced expression of MCP-1, hypoxia inducible factor-1, and plasminogen activator inhibitor-1 in cultured VSMCs was shown to be attenuated by transfection with antisense p22phox (15, 44), suggesting that ROS may be a common mediator of the inflammatory response.

PATHOPHYSIOLOGY OF ANG II DERIVED ROS

Ang II is involved in the pathogenesis of numerous cardiovascular diseases, including hypertension, atherosclerosis, restenosis, and cardiac hypertrophy. Recent work suggests that at least part of the mechanism by which Ang II influences the development of these diseases is via its ability to produce ROS.

Hypertension

Ang II is systemically or locally elevated in many forms of hypertension and is associated with increased oxidant stress in the kidney (as measured by thiobarbituric acid reactive substances protein carbonyl content) (53) and vascular $O_2^{\cdot-}$ production (13, 83, 103, 133). Ang II- but not norepinephrine-induced hypertension is accompanied by increased NAD(P)H oxidase activity in rat aortic media (103). In the same model, other cellular sources of $O_2^{\cdot-}$ remain unaffected, indicating a key role for the vascular NAD(P)H oxidases in Ang II-induced $O_2^{\cdot-}$ production *in vivo*. Indeed, mRNAs of two components of the smooth muscle enzyme, p22phox and nox-1 (37, and unpublished observations), as well as components of the adventitial oxidase, p67phox and gp91phox (21), are up-regulated in the rat aorta after 5–7 days of Ang II infusion. Increased expression of p67phox and gp91phox correlates with nitrotyrosine staining, another marker of oxidative stress (21).

The oxidase subunits expressed in hypertensive animals vary across the vessel wall. p22phox is evident in all layers of the wall (37, 91), whereas nox-1 expression is restricted to the media (unpublished observations). gp91phox, in contrast, is highly expressed in the adventitia and endothelium, but not in the medial layer (91). Experiments performed with gp91phox knock-out mice indicated that this subunit is not critical for basal $O_2^{\cdot-}$ production (129, 149), but rather is needed for Ang II-induced $O_2^{\cdot-}$ generation and hypertrophy (149). Although p47phox and p67phox are clearly present in the adventitia (91), their distribution in the other layers of the vessel wall has not been studied in hypertension.

Importantly, in animal models, Ang II-induced $O_2^{\cdot-}$ production and elevated blood pressure, as well as p22phox mRNA expression, are corrected by administration of SOD (13, 37) or the synthetic SOD mimetic tempol (85, 117). This blood pressure lowering effect of antioxidants suggests that increased oxidative stress precedes hypertension. However, in addition to the AT_1 receptor blocker losartan, the vasodilator hydralazine is also able to decrease blood pressure and $O_2^{\cdot-}$ production (37), which might be explained by activation of the NAD(P)H oxidase as a result of cyclic stretch (56). In fact, increased vascular $O_2^{\cdot-}$ production occurs not only in Ang II-induced hypertension, but also in models with depressed plasma renin activity. In comparison with wild-type animals, spontaneously hypertensive rats (SHR) have a significantly increased number of oxidized cells as detected by hydroethidine (133). Severe hypertension in Dahl salt-sensitive animals is linked with increased generation of $O_2^{\cdot-}$ in microvessels of the mesentery (134). Deoxycorticosterone acetate salt-induced hypertension similarly showed increased $O_2^{\cdot-}$ production in all layers of rat aorta (125). In another rat model of renal hypertension, "one kidney 1-clip"-induced high blood pressure was only lowered partially by tempol or losartan treatment, whereas their combination normalized it (28). Thus, both oxidative stress and the RAS may contribute to low renin forms of hypertension as well. As arteries that have been exposed to high blood pressure *ex vivo* (9) or by coarctation (39) have an induction of vascular ACE, it is likely that in these low plasma renin models, locally produced Ang II stimulates the NAD(P)H oxidases.

ROS have also been shown to play an important role in the pathology of the SHR. Administration of membrane-permeable SOD or tempol to SHR lowers blood pressure (83, 117), decreases renal vascular resistance (118), and blunts exaggerated tubuloglomerular feedback-dependent tone of the renal afferent arteriole (153). The exaggerated tubuloglomerular feedback is due to degradation of macula densa-derived NO by $O_2^{\cdot-}$ produced by Ang II and high blood pressure (152). Thus, Ang II and $O_2^{\cdot-}$ both contribute to the development of hypertension in SHR.

An involvement of oxidative stress in the pathology of essential hypertension in humans has also been reported (109, 112). Berry *et al.* (14) showed recently that human internal mammary arteries respond to Ang II perfusion by increased $O_2^{\cdot-}$ production. This process involves NAD(P)H oxidase induction and is AT_1 receptor-dependent. Moreover, the pressor effect of intrabrachial artery infusion of Ang II in humans is attenuated by coinfusion of the antioxidant vitamin C (27).

Restenosis

Restenosis remains the major limitation of coronary angioplasty. Many studies, primarily in the rat balloon angioplasty model, show that circulating or local activity of the RAS has a critical role in this process. In 1989, Powell *et al.* (98) demonstrated that the hyperproliferative response caused by balloon injury of rat carotid arteries can be reduced to ~20% if the animals are treated with the ACE inhibitor cilazapril, whereas two other antihypertensive drugs, verapamil and minoxidil, have no effect. Further investigation determined that Ang II infusion stimulates neointimal formation in response to injury (88), and that AT_1 blockers interfere with the process of neointimal formation (62, 86, 154). In addition, PD123319, a selective antagonist of the AT_2 receptor, also restricts the hyperproliferative response in pigs (154), demonstrating that Ang II can exert its effect on neointimal formation via both receptor subtypes. Local delivery of antisense ACE oligonucleotides to the site of injury attenuates neointimal formation in rat carotid arteries (81), suggesting a contribution of the tissue RAS to this hyperproliferative response.

In contrast to these findings, a role for Ang II in restenosis has not been supported by studies in knock-out animals or large animals or clinical trials. Harada *et al.* (52) found that balloon injury still induces intimal thickening in the AT_1 receptor knock-out mouse. It is possible that these transgenic animals up-regulate another aspect of the growth program to compensate for the loss of the RAS, but the identity of this putative signaling system remains unknown. Furthermore, administration of Ang II receptor antagonists has no effect on neointimal thickness in a porcine model of coronary artery restenosis (58). Similar results were found in a baboon model of restenosis using the ACE inhibitor cilazapril (51). Investigators of the MERCATOR and the MARCATOR clinical trials (30, 77) also concluded that ACE inhibition did nothing to prevent restenosis of the coronary artery. These studies were criticized for the doses used, the time of initiation of treatment, and the route of administration (99). These disparate findings underline the complexity of the progression of smooth muscle proliferation and vessel remodeling following vessel injury, and suggest that the RAS may contribute to this process, but is not itself sufficient to promote restenosis.

With this in mind, it is potentially important that Ang II induces $O_2^{\cdot-}$ production through vascular NAD(P)H oxidase activation *in vivo* and *in vitro*. $O_2^{\cdot-}$ has recently been identified as an important signaling molecule in several stages of the restenotic process. Increased oxidative stress is evident immediately after balloon injury (128) and 30 min later activates acute apoptotic cell death via redox-sensitive pathways (96). Ten to 14 days after balloon injury of pig coronary arteries or rat aorta, when neointimal cells are actively proliferating, $O_2^{\cdot-}$ production and lipid peroxidation are increased and glutathione peroxidase activity is decreased compared with uninjured arteries (40). Antioxidants, including vitamins C and E, reduce neointimal proliferation in several animal models (35, 40, 87), and human studies have shown a promising effect of the antioxidant probucol (for review, see 3). The oxidase stimulated by injury is DPI-inhibitable and NAD(P)H oxidase-dependent, suggesting that the NAD(P)H oxidases are activated in this process (128). Moreover, in the rat aorta balloon injury model, p47phox protein is increased after injury and is localized to the neointima and media of injured arteries (93). In addition, injury-induced $O_2^{\cdot-}$ production is associated with augmented NAD(P)H oxidase activity and up-regulation of p47phox and p67phox in adventitial fibroblasts (122). We recently found increased expression of p22phox and nox-1 3–15 days after injury (135), supporting the conclusion that NAD(P)H oxidases are involved in restenosis. Future work using knock-out animals will be required to define the role of NAD(P)H oxidases in this pathological response.

Atherosclerosis

Ang II has also been implicated in the pathogenesis of experimental atherosclerosis. In Watanabe heritable hyperlipidemic rabbits, 9 months of orally administered captopril decreases aortic atherosclerotic lesion formation (20). Subsequent clinical studies showed that ACE is increased in atherosclerotic plaques of human coronary arteries (26). Another potential source of Ang II in atherosclerotic arteries is activated macrophages (97), which also produce ROS.

The direct effect of Ang II on atherogenesis was studied in $ApoE^{-/-}$ mice. Two groups demonstrated that prolonged Ang II infusion dramatically accelerates lesion formation in this animal model (24, 151). Furthermore, AT_1 receptor blockade decreases lesion area and NADH-driven $O_2^{\cdot-}$ production in intact aortic rings from Watanabe hypercholesterolemic rabbits (150), suggesting that the mechanism by which Ang II promotes atherosclerosis may involve induction of ROS. These ROS may influence atherogenesis not only by oxidizing low-density lipoprotein, but also by serving as signaling molecules to promote VSMC migration and hypertrophy.

NAD(P)H oxidases within the vessel wall may also be functionally important for atherogenesis. p22phox expression is increased in all layers of atherosclerotic human coronary arteries in both phagocytic and nonphagocytic cells (4). gp91phox is expressed intensely in macrophage-rich areas of the lesion, whereas nox-4 expression is increased in neointimal areas rich in α-actin-positive cells (126). In the $ApoE^{-/-}$

mouse aorta, p47phox is expressed throughout the wall, and is associated with increased $O_2^{\cdot -}$ generation and lesion formation in response to a high fat diet (10). This widespread up-regulation of NAD(P)H oxidase components suggests that further investigation of the role of these enzymes in atherogenesis is warranted.

Renal disease

Accumulating evidence indicates that ROS are important mediators of the progression of renal diseases such as acute renal failure (84), immune and nonimmune glomerular diseases, tubulointerstitial diseases (65), and diabetic nephropathy (113). In the inflammatory renal diseases, ROS are produced mainly by invading inflammatory cells such as macrophages and neutrophils stimulated by particulate and soluble factors. On the other hand, resident kidney cells (glomerular mesangial cells, glomerular podocytes, proximal tubule epithelial cells, *etc.*) may be sources of ROS in noninflammatory kidney diseases.

Interestingly, blockade of Ang II effects by ACE inhibitors or AT_1 antagonists has been shown to dramatically attenuate the decline in renal function associated with several forms of chronic renal disease (136). Ang II has the ability to activate inflammatory cells by mediating direct chemotaxis and production of proinflammatory mediators such as MCP-1 and transforming growth factor-β (108), perhaps via the production of ROS. As many factors in addition to Ang II may be critically involved in the production of ROS in renal diseases, the correlation between Ang II and the production of ROS requires further investigation.

CONCLUSIONS AND FUTURE DIRECTIONS

The recent HOPE trial clearly demonstrated that Ang II has pathophysiological effects that are in addition to its pressor effects (159). The work cited in this review suggests that the ability of Ang II to induce ROS, and the consequent effects on endothelial function, smooth muscle growth, and inflammatory responses, may in part account for these additional effects of Ang II. Investigation into the mechanisms by which Ang II increases ROS formation, the molecular targets of ROS, and the development of animal models in which to test some of these hypotheses will lead to exciting advances in this important area.

ABBREVIATIONS

ACE, angiotensin converting enzyme; Ang II, angiotensin II; AP-1, activator protein-1; AT_1, angiotensin type 1; DPI, diphenylene iodonium; EGFR, epidermal growth factor receptor; ERK 1/2, extracellular signal-regulated kinase; FAK, focal adhesion kinase; H_2O_2, hydrogen peroxide; IκB, inhibitor of κB; JAK2, janus kinase 2; JNK/SAPK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; NO, nitric oxide; $O_2^{\cdot -}$ superoxide; PDGF, platelet-derived growth factor; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PMA, phorbol myristate acetate; PYK2, proline-rich tyrosine kinase 2; RAS, renin–angiotensin system; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; STATs, signal transducer and activator of transcription; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular smooth muscle cell.

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