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Interactions of angiotensin II with NAD(P)H oxidase, oxidant stress and cardiovascular disease

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Abstract

An elevation in angiotensin II (Ang II) levels is a common occurrence in a diverse number of cardiovascular diseases including hypertension, hypercholesterolaemia, atherosclerotic coronary artery disease, left ventricular hypertrophy (LVH), heart failure and diabetes. An important effect of Ang II is activation of the NAD(P)H oxidase, a major source of reactive oxygen species (ROS) production by vascular cells. This increase in cellular ROS contributes to the pathogenesis of vascular disease by altering endothelial cell function, enhancing smooth muscle cell growth and proliferation, stimulating inflammatory proteins, including macrophage chemoattractant agents, growth factors and cytokines, and modulating matrix remodelling. Studies of genetically-altered mice have unequivocally shown that activation of the NAD(P)H oxidase by Ang II contributes to hypertension, LVH and atherosclerosis. Furthermore, increasing evidence suggest that the NAD(P)H oxidase contributes to human disease, suggesting that it is a potential target for future therapeutic intervention.

Introduction

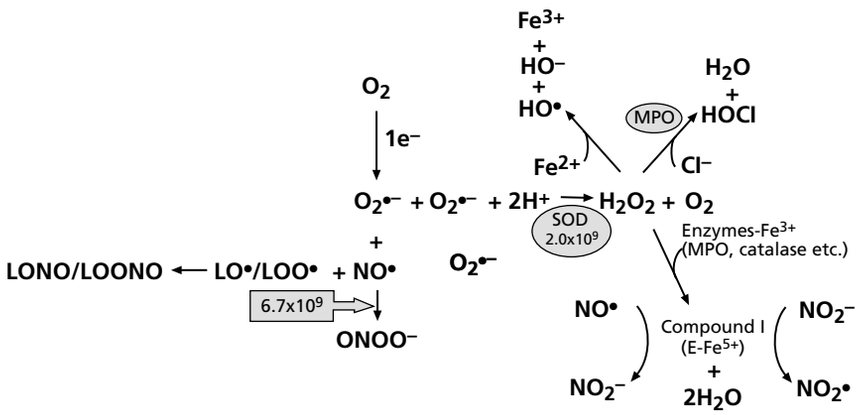
A large body of evidence has demonstrated a significant role for angiotensin II (Ang II) in the pathogenesis of numerous cardiovascular diseases including hypertension, hypercholesterolaemia, atherosclerotic coronary artery disease, left ventricular hypertrophy (LVH), heart failure and diabetes. In these conditions, inflammatory cells that enter the vessel wall express angiotensin-converting enzyme (ACE) and serve as sites for excessive tissue production of Ang II. Activation of the AT_1-receptor by Ang II leads to a variety of intracellular signalling events, ultimately causing dysfunction of the endothelium, proliferation of vascular smooth muscle cells, expression of pro-inflammatory genes, and reconstruction of extracellular matrix. These molecular events lead to vasoconstriction, inflammation, vascular and cardiac hypertrophy, atherosclerotic plaque formation and rupture and are translated clinically into hypertension, coronary ischaemia, heart failure and stroke. Many of the untoward effects of Ang II seem to be mediated by the production of reactive oxygen species (ROS), which in turn contribute to virtually all of the disease processes described above. In this review, we will explore the interactions between Ang II, the NAD(P)H oxidase and ROS in terms of their ability to modulate cardiovascular disease.

Reactive oxygen species, antioxidant defences and oxidant stress

In the process of normal cellular metabolism, several enzymes and enzyme systems are capable of generating or accepting electrons, leading to the production of ROS (Figure 1). The one-electron reduction product of molecular oxygen is superoxide ($O_2^{\bullet-}$), which in turn serves as a progenitor for numerous other ROS.[1] Superoxide is converted to hydrogen peroxide (H_2O_2) spontaneously or is catalysed to H_2O_2 by superoxide dismutase (SOD, see below).[2] Many ROS possess unpaired electrons and thus are free radicals. These include molecules such as $O_2^{\bullet-}$, hydroxyl racial ($HO^{\bullet}$), nitric oxide ($NO^{\bullet}$), and lipid radicals ($LO^{\bullet}$ and $LOO^{\bullet}$). Of these, $NO^{\bullet}$ and higher oxides of $NO^{\bullet}$ are also referred to as reactive nitrogen species. Other ROS, such as H_2O_2, peroxynitrite (ONOO-), and hypochlorous acid (HOCl), are not free radicals *per se*, but have oxidising effects that contribute to oxidant stress. The chemistry of the formation of these radicals and other reactions discussed below are illustrated in Figure 1.

Very important to vascular biology is the fact that $O_2^{\bullet-}$ and $NO^{\bullet}$ react with one another at a near diffusion-limited rate.[3] This reaction leads to the formation of the above-mentioned peroxynitrite anion, which is an extremely strong oxidant. Superoxide is not the only radical that can react with $NO^{\bullet}$. Lipid radicals ($LO^{\bullet}$ and $LOO^{\bullet}$ mentioned above), can react with $NO^{\bullet}$ to form LONO and LOONO respectively.[4] It is of interest that oxidised low-density lipoprotein (LDL), but not native LDL, added to isolated vessels inhibits endothelium-dependent vascular relaxation.[5] The oxidation of LDL leads to production of linoleic hydroperoxy- and alkoxy-radicals that could participate in such reactions with $NO^{\bullet}$. It has also been shown that hydroxyl radicals may react with $NO^{\bullet}$.[6] Finally, mammalian peroxidases, such as myeloperoxidase (MPO) and the eosinophil peroxidase, upon reaction

Figure 1 Chemistry of reactive oxygen species (ROS) formation



with H_2O_2 form a highly reactive π-radical, commonly referred to as compound I, which can oxidise NO• to NO_2^- and react with NO_2^- to form NO_2•. NO_2• can in turn participate in nitrating events, such as formation of nitrotyrosines.[7,8] Even in the presence of $O_2^{\bullet-}$, MPO can increase consumption of NO•.[9] This is potentially very important, because MPO can be released by inflammatory cells in the vessel wall and is taken up by endothelial cells, where it can impair endothelium-dependent vasodilatation.[8] Oxidative inactivation of NO• has been shown to occur in numerous pathological entities, including atherosclerosis, hypertension, diabetes and cigarette smoking, and almost certainly contributes to the pathology of these conditions.[10,11]

The cellular production of one ROS may lead to the production of others via radical chain reactions. For example, reactions between radicals and polyunsaturated fatty acids within the cell membrane may result in a fatty acid peroxyl radical (R-COO•) that can attack adjacent fatty acid side chains and initiate production of other lipid radicals. Lipid radicals produced in this chain reaction accumulate in the cell membrane, and have myriad effects on cellular function, including leakage of the plasmalemma and dysfunction of membrane-bound receptors. Of note, end-products of lipid peroxidation, including unsaturated aldehydes and other metabolites have cytotoxic and mutagenic properties.[12] Another example is the reaction of H_2O_2 with peroxidases mentioned above.

Because of the necessity of existing in an oxygen-rich environment which promotes the formation of ROS, both prokaryotic and eukaryotic cells possess numerous antioxidant defence mechanisms. These include small molecule antioxidants such as ascorbic acid, glutathione, uric acid, bilirubin, and tetrahydrobiopterin. In addition, mammalian cells have developed a number of enzymes that are quite effective at removing radicals. The SODs, which catalyse the conversion of $O_2^{\bullet-}$ to H_2O_2 and O_2, include the cytoplasmic Cu/Zn SOD or SOD1, the mitochondrial MnSOD or SOD2, and the extracellular SOD (ecSOD or SOD3). The latter enzyme is of interest because it is highly expressed in blood vessels. It is produced by vascular smooth muscle cells, fibroblasts and macrophages and is secreted into the extracellular matrix, where it binds to heparin-binding sites on glycosaminoglycans.[13,14] The rate of reaction between $O_2^{\bullet-}$ and the SOD occurs at a rate exceeding 10^9 M^{-1}·sec^{-1}, a rate that approaches that of the reaction between $O_2^{\bullet-}$ and NO•. Indeed, in compartments in which $O_2^{\bullet-}$, NO• and SOD co-exist, there is competition between NO• and SOD for the reaction with $O_2^{\bullet-}$. Modest changes in SOD concentrations or activity may therefore greatly affect the biological activity of NO• by preventing its oxidative degradation. Mammalian cells also possess both catalase and glutathione peroxidase which react with H_2O_2. In the case of catalase, the product of this reaction is simply O_2 and H_2O_2. In mammalian cells, glutathione peroxidase has become a predominant mechanism for metabolism of H_2O_2. This enzyme uses glutathione as a co-factor and the reaction of glutathione, H_2O_2 and glutathione peroxidase leads to formation of oxidised GSSG and H_2O. This dependence on glutathione is important because peroxide stresses may serve to deplete cells of glutathione, and treatment with glutathione precursors such as cysteine, N-acetylcysteine (NAC) or the pro-cysteine agent L-2-oxothiazolidine-4-carboxylate (OTZ) can ameliorate this effect. Probably most of the antioxidant effects of cysteine or NAC are based on their ability to enhance intracellular glutathione levels, permitting full function of glutathione peroxidase. Other intracellular antioxidant proteins include thioredoxin and glutaredoxin. These sulfhydryl-containing proteins react with peroxides including lipid hydroperoxides and H_2O_2.[15,16] By reducing protein disulphide bonds, these small proteins also modulate the DNA binding activities of the redox-sensitive transcription factors, such as

nuclear factor kappa B (NF-κB), AP-1, redox factor-1 (REF-1) and hypoxia-induced factor 1 (HIF-1) to affect redox signalling.[17] Under normal circumstances, these antioxidant defences are sufficient to keep ambient ROS levels at a minimum. In the setting of several pathological conditions, however, the production of ROS may increase to outstrip the antioxidant defence mechanisms, upsetting this tenuous balance and leading to cellular oxidant stress.

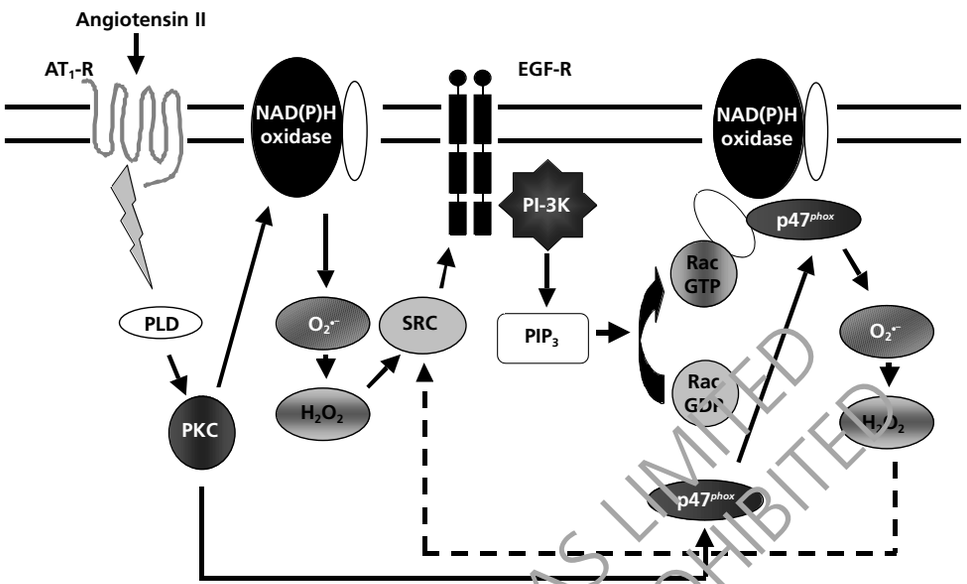
While ROS are often considered toxic products leading to disease, there is evidence that they may play important roles in cell signalling. H_2O_2 in particular, functions as a signalling intermediate for many pathophysiological responses.[18] Early work demonstrated that H_2O_2 can both activate tyrosine kinases and inhibit tyrosine phosphatases.[19,20] Mitogen activated protein kinase (MAPK) family members ERK1/2, p38 MAPK, ERK5 and JNK have been shown to be phosphorylated and activated by H_2O_2.[21-25] H_2O_2 also mediates epidermal growth factor (EGF)–induced phosphorylation of its receptor and phospholipase C,[26] platelet-derived growth factor (PDGF) stimulation of STAT kinases JAK2 and TYK2,[27] activation of Akt by Ang II,[28] and tyrosine phosphorylation of protein kinase C.[29] The precise mechanism by which H_2O_2 activates these protein kinases is unclear, but could involve direct modification of inactive cysteines or regulation of phosphatases leading indirectly to increased tyrosine phosphorylation of these enzymes. Activation of Ras by reactive oxygen species is also potentially quite important.[30] Furthermore, it has been shown that catalase, after reacting with H_2O_2, can activate guanylate cyclase.[31] This seems to occur via a unique mechanism that is quite different from the haeme-mediated activation of guanylate cyclase by NO•.[31] Recent data suggest that H_2O_2 can activate PI 3-kinase leading to phosphorylation of endothelial NO• synthase (eNOS) at serine 1179, which may contribute to the activation of eNOS in response to Ang II.[25,32,33]

Vascular sources of ROS – role of the NAD(P)H oxidases

In mammalian cells, potential enzymatic sources of ROS include the mitochondrial electron transport chain, the arachidonic acid metabolising enzymes lipoxygenase and cycloxygenase, the cytochrome P450s, xanthine oxidase, NAD(P)H oxidases, nitric oxide synthase (NOS), peroxidases, and other haemoproteins. While all of these may be important under certain circumstances and in various tissues, it is now generally recognised that the NAD(P)H oxidases play a very important role in the production of ROS in vascular cells. In numerous studies, investigators have attempted to define the source of ROS using homogenates of either vascular cells or tissues. In such experiments, the relevant enzymes are characterised based on their substrate preference and, in some cases, specific inhibitors. When homogenates of endothelial and vascular smooth muscle cells have been studied in this fashion, the predominant substrates capable of driving $O_2^{\bullet-}$ production have been NADH and NADPH, no matter what detection system has been employed. In fact, in these studies, substrates for other enzyme systems, such as xanthine for xanthine oxidase, arachidonic acid for cyclooxygenase, or cytochrome P450s or succinate for mitochondrial electron transport, have been ineffective in stimulating $O_2^{\bullet-}$ in cellular homogenates. As an example, using electron spin resonance (ESR) for $O_2^{\bullet-}$ detection, we recently demonstrated robust NADPH and NADH oxidase activity in cultured endothelial and smooth muscle cells.[34,35] A caveat with these studies is that the assay systems may not have been optimum for demonstrating $O_2^{\bullet-}$ production from some of these other sources. Another consideration is that these assays may not be specific for the classical NAD(P)H oxidase. For example, xanthine oxidase may use NADH as a substrate for $O_2^{\bullet-}$ production. Nevertheless, based on such studies, it has been proposed that the predominant $O_2^{\bullet-}$-producing enzymes in the endothelium and vascular smooth muscle are NAD(P)H oxidases. Studies using antisense oligonucleotides against specific subunits of the NAD(P)H oxidase and studies of cells and vessels from genetically altered mice have further confirmed that this enzyme family is very important in vascular tissues (discussed more in depth below).

The structure and function of the NAD(P)H oxidase was initially well characterised for neutrophils and other professional phagocytic cells. In phagocytic cells, two membrane components, $p22^{phox}$ and $gp91^{phox}$, comprise the cytochrome b558, which is regulated by cytoplasmic subunits, including $p47^{phox}$, $p67^{phox}$ and the small G-protein Rac. Upon stimulation, the cytosolic subunits assemble with the membrane cytochrome to form a functioning oxidase that uses NADPH as an electron donor, resulting in the well characterised oxidative burst. On a molecular level, the vascular oxidases share limited homology with the neutrophil respiratory burst oxidase. Many of the neutrophil components, including $p22^{phox}$, $p47^{phox}$ and the small GTPase Rac, are present in vascular cells. During the past few years, a family of proteins termed the Nox proteins, with homology to the neutrophil oxidase catalytic subunit, $gp91^{phox}$, has been discovered and shown to play a critical role in function of both the smooth muscle and endothelial cell NAD(P)H oxidases.[36-38] The Nox proteins seem to have evolved from a common ancestral protein, and the originally described $gp91^{phox}$ has now been reclassified as Nox2. Endothelial cells contain Nox1, Nox2 and Nox4, while vascular smooth muscle cells express Nox1, Nox4 and Nox5.[38,39] Importantly, the Nox proteins contain binding sites for NAD(P)H, flavins and haeme, and therefore may function independently of the other subunits in formation of $O_2^{\bullet-}$, although there is strong evidence that the other subunits contribute to full function of the oxidase (see below). Very recently, two newly identified proteins with homology to $p47^{phox}$ and $p67^{phox}$ have been cloned and shown to regulate Nox1 activity,

Figure 2 Signal transduction mechanisms of angiotensin II (Ang II) activation of the vascular NAD(P)H oxidase. Ang II activates the NAD(P)H oxidase in a biphasic fashion. The initial activation of the oxidase is dependent on protein kinase C (PKC). H_2O_2 produced from the oxidase activates c-Src which transactivates epidermal growth factor receptor (EGFR), leading to PI 3-kinase activation. Production of PIP3 activates Rac-1, causing assembly and activation of the NAD(P)H oxidase for the second phase activation, accompanied by greater H_2O_2 production. PKC may partially be involved in the second phase activation via phosphorylation and translocation of the $p47^{phox}$. The sustained increase in H_2O_2 feeds forward to further c-Src activation and subsequently prolonged activation of the NAD(P)H oxidase



suggesting that multiple homologues of the cytosolic proteins may modulate enzyme activity in tissue or stimulus-specific fashions.[40]

The vascular NAD(P)H oxidases differ from the neutrophil oxidase in many respects. The neutrophil oxidase releases massive amounts of $O_2^{\bullet-}$ in bursts, while the vascular oxidases produce $O_2^{\bullet-}$ at low levels in a continuous fashion. In rat aortic smooth muscle cells, Nox1 has been shown to localise at the cell surface, while Nox4 is present in focal adhesions, where it co-localises with $p22^{phox}$.[41] Other recent preliminary data from our laboratories have shown that in vascular smooth muscle cells Nox1 and $p22^{phox}$ directly interact in a fashion similar to that observed in neutrophils.[42]

Another interesting difference between the vascular and phagocytic oxidases relates to the ROS formed. Both of these enzymes contain a haeme group localised within a pore that seems responsible for reduction of oxygen. Since the Fe^{2+} can only perform a one-electron oxidation of oxygen, it follows that the predominant product of these enzymes should be $O_2^{\bullet-}$. Indeed this is the case for the neutrophil oxidase. In contrast, recent data from our laboratories have shown that, while the vascular NAD(P)H oxidases produce $O_2^{\bullet-}$, they produce approximately four-fold greater amounts of H_2O_2. It is possible that the release of $O_2^{\bullet-}$ from the vascular Nox proteins is hindered by charge within the haeme-containing pore. H_2O_2, formed by spontaneous dismutation of $O_2^{\bullet-}$, would not be subject to such hindrance due to its lack of charge.

Regulation of the vascular oxidases by pathological stimuli – role of angiotensin II

The activity of the vascular NAD(P)H oxidases is regulated by cytokines, hormones, and mechanical forces that are known to be involved in the pathogenesis of vascular disease. Stimulation of vascular smooth muscle cells with thrombin, platelet-derived growth factor, tumour necrosis factor-α, and lactosylceramide all increase activity of the vascular ROS formation and NAD(P)H oxidase activity.[43-46] Exposure of human umbilical endothelial cells to 5 or 20 dyne/cm^2 unidirectional laminar shear stress results in a transient elevation in NADH-dependent $O_2^{\bullet-}$ formation, whereas oscillatory shear caused a sustained increase in oxidase activity.[47] Both the endothelial and vascular smooth muscle oxidases have also been reported to be activated by cyclic stretch.[48-50]

Accumulating evidence has shown that Ang II is an extremely important stimulus for activation of the vascular NAD(P)H oxidase. Griendling *et al*.[51] first demonstrated that nanomolar concentrations of Ang II very potently stimulated NAD(P)H oxidase activity in cultured vascular smooth muscle cells. Recent work by several groups has provided insight into how the NAD(P)H oxidase is activated by this hormone. Studies of vascular smooth muscle cells by Seshiah *et al*.[52] demonstrate that this response is biphasic, with an initial peak of H_2O_2 production occurring at 30 seconds. This is due to AT_1-receptor-dependent, PKC-mediated activation of the NAD(P)H oxidase (Figure 2).

This activation of PKC leads to phosphorylation of $p47^{phox}$,[53] which then associates with the membrane components of the oxidase. The initial production of H_2O_2 leads to stimulation of c-Src, evoking EGF receptor transactivation and production of PIP_3 by PI3 kinase. PIP_3, in turn, activates the small molecular weight GTPase Rac-1, which also associates with the membrane complex, leading to a second phase activation of the NAD(P)H oxidase. This second phase activation of the oxidase leads to increased production of $O_2^{\bullet-}$ and H_2O_2 at 30 minutes. Interestingly, H_2O_2 can activate c-Src through this feed forward mechanism to maintain the activity of the vascular smooth muscle NAD(P)H oxidase.

As discussed above, there is unequivocal evidence that the cytosolic subunit $p47^{phox}$ is important in oxidase activation. Neither vascular smooth muscle,[54] nor endothelial cells,[55,56] from $p47^{phox-/-}$ mice are able to produce $O_2^{\bullet-}$ in response to Ang II. Recent work from Li *et al*. indicates that, in human microvascular endothelial cells, $p47^{phox}$ is phosphorylated on serine within 1 minute of Ang II exposure.[56] The authors demonstrate that this is followed by a translocation of $p47^{phox}$ to nuclear and membrane fractions and an increased association of $p47^{phox}$ with $p22^{phox}$.[56] A very recent study suggests c-Src is required for the rapid $p47^{phox}$ phosphorylation and its recruitment to the membrane, as well as $O_2^{\bullet-}$ production in vascular smooth muscle cells in response to Ang II.[57] Thus, $p47^{phox}$ clearly plays a critical role in the ability of the NAD(P)H oxidase to respond to hormonal stimulation. The basal production of ROS by endothelial cells may not require $p47^{phox}$, as it does not seem to be impaired in cells from $p47^{phox-/-}$ mice. This is compatible with the concept discussed above that the Nox proteins have all the components necessary for electron transfer from NAD(P)H to oxygen to form $O_2^{\bullet-}$. The role of $p47^{phox}$ and probably Rac 1 seems to be to enhance activity of the enzyme complex in response to hormonal stimuli such as Ang II.

Interestingly, Ang II not only increases activity of the NAD(P)H oxidase, but over the long-term also increases expression of its subunits. The aortic mRNA expression of $p22^{phox}$, Nox1 and Nox4 are all increased during chronic Ang II infusion in rats.[58,59] In preliminary studies, we have produced mice overexpressing $p22^{phox}$ in the vascular smooth muscle cells and find that the aortae of these animals have increased expression of Nox1 and Nox4. This seems to suggest that an increase in one subunit of the NAD(P)H oxidase can lead to upregulation of its binding partners to modulate the activity of the oxidase.

Events downstream of ROS, angiotensin II and NAD(P)H oxidase activation

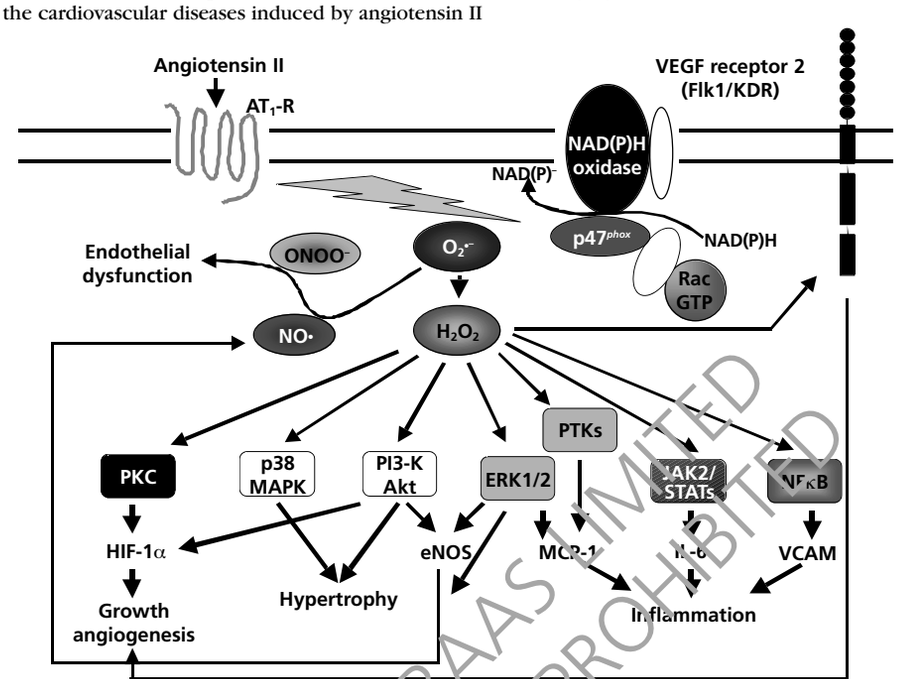
Once the NAD(P)H oxidase is activated, the ROS produced lead to a plethora of cellular events that are modulated by the degree and duration of oxidase activation, as well as other autocrine and paracrine stimuli. It is now clear that H_2O_2 generated from the NAD(P)H oxidase is essential for the vascular smooth muscle cell hypertrophy caused by Ang II. Stable transfection of vascular smooth muscle cells with antisense $p22^{phox}$ sequence prevented H_2O_2 generation in response to AT_1-receptor activation and reduced new protein synthesis.[60,61] Stable expression of catalase had a similar effect, supporting a role for H_2O_2 in the hypertrophic response to Ang II. Downstream signals activated by H_2O_2 include both p38 MAP kinase and PI 3-kinase/Akt, and inhibition of these pathways abrogates the hypertrophic response to Ang II.[23,28] It was reported that Ang II activation of ERK1/2 in vascular smooth muscle also requires H_2O_2.[62]

In addition to hypertrophy, Ang II stimulates cellular inflammatory responses via redox-sensitive mechanisms. Tummala *et al*.[63] demonstrated that Ang II increased binding activity of NFκB and transactivated a NFκB-driven vascular cell adhesion molecule-1 (VCAM-1) promoter in vascular smooth muscle cells. Importantly, the tyrosine kinases JAK2/STATs are activated by Ang II and this response can be prevented by inhibition of the NAD(P)H oxidase by electroporation of an antibody against $p47^{phox}$.[64] This activation of JAK2/STATs is important for interleukin-6 (IL-6) upregulation in vascular smooth muscle, supporting a critical role for redox-sensitive signalling events in inflammation caused by Ang II. The expression of monocyte chemoattractant protein-1 (MCP-1) is also increased by Ang II in vascular smooth muscle cells.[65] This response seems to require ERK1/2 MAP kinase and tyrosine kinase activation induced by NAD(P)H oxidase-derived H_2O_2, as the pharmacological kinase inhibitors, DPI and catalase, prevent MCP-1 upregulation by Ang II.[65] NAD(P)H oxidase activation is also important for upregulation of intercellular adhesion molecule-1 (ICAM-1) in response to TNFα.[66] A very recent study has shown that $p47^{phox}$ activation is important for NFκB transactivation.[67]

A third vascular response modulated by ROS generated by the NAD(P)H oxidase is angiogenesis. Ushio-Fukai *et al*. have shown that the NAD(P)H oxidase-derived ROS mediates vascular endothelial cell growth factor (VEGF) receptor transactivation.[68] Likewise, Ang II activation of growth factor receptors, such as epidermal growth factor receptor (EGFR), is also dependent on H_2O_2 production.[69] Additional studies have demonstrated that induction of HIF-1α and VEGF by Ang II is redox sensitive.[70]

Finally, we and others have found that H_2O_2 potently activates the eNOS via both calcium-dependent and independent mechanisms.[25,33] In a subsequent study, we showed that endogenous H_2O_2, produced by the NAD(P)H oxidase in response to Ang II, activates eNOS to produce $NO^{\bullet}$.[32] In this study, we found that endothelial cells from $p47^{phox-/-}$ mice were unable to produce $NO^{\bullet}$ in response to Ang II, but generated $NO^{\bullet}$ in a manner similar to wild-type cells when stimulated with the calcium ionophore, A23187, or bradykinin. Thus, Ang II has the unique property of almost simultaneously stimulating production of $O_2^{\bullet-}$ and $NO^{\bullet}$.

Figure 3 Downstream signalling events of NAD(P)H oxidase activation by angiotensin II (Ang II). Ang II activates the vascular NAD(P)H oxidases, leading to $O_2^{\bullet-}$ production. Superoxide is dismutated to H_2O_2, which mediates regulation of a variety of pro-inflammatory genes including MCP-1, IL-6 and vascular cell adhesion molecule (VCAM). Activation of the p38 mitogen-activated protein kinase (MAPK), PI3-kinase/Akt and ERK1/2 pathways by Ang II mediates hypertrophic responses. H_2O_2 also activates endothelial NO synthase (eNOS) via ERK1/2 and Akt to produce NO•. By activating protein kinase C (PKC) and utilising PI3-kinase, H_2O_2 increases the expression of HIF-1α, leading an increase in vascular endothelial cell growth factor (VEGF) expression and subsequently vascular cell growth and angiogenesis. H_2O_2-dependent activation of VEGF receptor 2 (Flk1/KDR) also contributes to angiogenesis. These mechanisms at least partially account for the cardiovascular diseases induced by angiotensin II



This may explain prior studies showing that Ang II stimulates $ONOO^-$ in endothelial cells.[71,73]

These downstream signalling events and other responses discussed below, stimulated by Ang II activation of the vascular NAD(P)H oxidases, are summarised schematically in Figure 3.

Studies of the vascular NAD(P)H oxidase in vivo

The functional significance of NAD(P)H oxidase activation by Ang II *in vivo* has been extensively studied in experimental animals. In rats made hypertensive by chronic Ang II infusion, vascular $O_2^{\bullet-}$ production is dramatically increased, as is NAD(P)H oxidase activity.[58,74,75] Blood pressure (BP) and vascular reactivity are restored by exogenous liposome-encapsulated SOD in those rats.[76] Recently, we and others have found further evidence that NAD(P)H oxidase is the major source of $O_2^{\bullet-}$ in this model of hypertension using genetically modified mice.[55,77] Mice deficient in the $p47^{phox}$ subunit of the oxidase do not increase $O_2^{\bullet-}$ production in response to Ang II, as is the case with vascular cells cultured from these animals. Importantly, the hypertension caused by Ang II is rather dramatically diminished in $p47^{phox-/-}$ mice as compared with C57BL/6 mice.

The NAD(P)H oxidase contributes to forms of hypertension other than that induced by Ang II. Zalba and colleagues recently reported that NAD(P)H oxidase-derived $O_2^{\bullet-}$ production is increased in SHR.[78] We have recently found that deoxycorticosterone acetate (DOCA)-salt hypertension is associated with increased oxidation of tetrahydrobiopterin within the endothelium. Tetrahydrobiopterin is a critical co-factor for the NOS, and in its absence, the NOSs produce $O_2^{\bullet-}$ rather than NO•, a condition referred to as NOS uncoupling. In keeping with this, we found that DOCA-salt hypertension is associated with a marked increase of eNOS-derived $O_2^{\bullet-}$.[79] In $p47^{phox-/-}$ mice made to have DOCA-salt hypertension, tetrahydrobiopterin oxidation was much less than that observed in C57BL/6 mice with hypertension, and eNOS uncoupling did not occur. These data suggest that hypertension leads to a cascade of events in which the NAD(P)H oxidase is activated, leading to oxidation of tetrahydrobiopterin, and production of large amounts of $O_2^{\bullet-}$ from uncoupled eNOS. Of interest, in this recent study, we found that oral administration of tetrahydrobiopterin was very effective in 're-coupling' of eNOS, i.e., reducing eNOS-derived $O_2^{\bullet-}$ production and increasing endothelial NO• production.[79] Uncoupling of eNOS has been observed in atherosclerosis,[80] diabetes,[81] and insulin resistant states,[82] and it is interesting to speculate that initial activation of the NAD(P)H oxidases may underlie this phenomenon in these conditions and that treatment with tetrahydrobiopterin or efforts to prevent tetrahydrobiopterin oxidation may be useful.

It is generally assumed that the mechanism

responsible for hypertension caused by increased production of ROS relates to oxidative inactivation of endothelial-derived NO•, resulting in a loss of vasodilatation, increased systemic vascular resistance and thus hypertension. While this may be correct, there are other mechanisms that may come into play. For example, Zimmerman *et al*.[83] have recently shown that intraventricular Ang II administration increases ROS production in the circumventricular organs and that adenoviral overexpression of SOD in the circumventricular organs completely prevented the hypertensive response to intraventricular administration of Ang II. Another example of how ROS may increase BP is desensitisation of the carotid baroreflex. Li *et al*.[84] has shown that the carotid baroreflex is much less responsive in atherosclerotic rabbits, and that this can be corrected by administration of catalase into the carotid sinus. Finally, the NAD(P)H oxidase subunits have all been identified in renal tubular cells, which are responsive to Ang II.[85,86] It is quite possible that oxidative stress induced by Ang II in the kidney could contribute to hypertension via effects independent of the vasculature.

In addition to hypertension, the vascular NAD(P)H oxidases are responsible for excessive $O_2^{\bullet-}$ generation in other cardiovascular diseases in which tissue Ang II levels are increased. Probably the most important of these is atherosclerosis. The NAD(P)H oxidase is activated and vascular $O_2^{\bullet-}$ production is increased in atherosclerotic vessels of rabbits with experimental atherosclerosis, and this is corrected by Ang II receptor blockade.[87] In segments of human saphenous veins obtained from patients undergoing routine coronary artery bypass surgery, Guzik and colleagues reported that both diabetes and hypercholesterolaemia are associated with increased NADH-dependent $O_2^{\bullet-}$ production.[88] Importantly, the progression of atherosclerosis is diminished in Apo(E)-deficient mice that have been crossed with $p47^{phox-/-}$ mice.[89] It has recently been reported that the NAD(P)H oxidase activity and the expression of its $p47^{phox}$ and $p22^{phox}$ subunits are increased in atherosclerotic abdominal aortic aneurysms of humans.[90] We have recently used ESR assays to show that NAD(P)H oxidase activity is increased in human atherosclerotic coronary arteries.[91]

Two other conditions in which there is strong evidence for a role of the NAD(P)H oxidase are heart failure and nitrate tolerance. In rats with heart failure secondary to chronic myocardial infarction (MI), both basal and NADH-stimulated $O_2^{\bullet-}$ production is significantly elevated.[92] Treatment with SOD markedly improved endothelium-dependent vasorelaxation in vessels of these animals.[92] In nitrate tolerance, the activity of the NAD(P)H oxidase is increased.[93] The vascular relaxation of nitrate-tolerant vessels can be corrected by treatment with membrane permeable forms of SOD,[93] and also by treatment with angiotensin receptor antagonists.[94]

Antioxidants and the treatment of cardiovascular disease

Given the overwhelming evidence that oxidative stress is associated with, and in fact contributes to, a diverse number of cardiovascular diseases, it would seem logical that antioxidants would improve these conditions. Several observational population studies have suggested that this is the case. In many of these studies, the intake of high amounts of antioxidant vitamins in the diet is associated with reduced cardiovascular disease.[95] There are a few prospective studies that suggest that antioxidant vitamins may prevent disease. In the recent Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) trial, the progression of carotid intimal/medial thickness was monitored in subjects treated with either placebo, vitamin E alone, vitamin C alone, or the combination of these agents.[96] Compared with placebo, neither vitamin E nor vitamin C alone affected progression of carotid intimal/medial thickness. In contrast, the combination of the two reduced this process by half.[96] In the Cambridge Heart Antioxidant Study (CHAOS), vitamin E was found to significantly reduce non-fatal MI and major cardiovascular events, but had no effect on cardiovascular deaths.[97] More recently, in a small study of 37 patients who received heart transplants, Fang *et al*.[98] used intravascular ultrasound to follow the progression of transplant-related atherosclerosis. The investigators showed that the combination of vitamin E and vitamin C markedly inhibited this process.[98]

Despite the promising information from observational cohort studies and the above mentioned small randomised trials, several large prospective studies have failed to show any benefit from treatment with antioxidant vitamins. In the Heart Outcomes Prevention (HOPE) Trial, vitamin E was found to have no effect on major cardiovascular events compared with placebo in >9,500 high-risk subjects.[99] In the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI) prevention trial, >11,000 MI survivors were randomised to receive n-3 polyunsaturated fatty acids (PUFA), vitamin E, neither, or both.[98] While the PUFA therapy reduced cardiovascular event rates significantly, vitamin E treatment had only an insignificant effect.[100] In the study to evaluate carotid ultrasound changes in subjects treated with ramipril or vitamin E (SECURE) substudy of the HOPE trial, vitamin E was ineffective in preventing progression of carotid intimal/medial thickness, while the ACE inhibitor, ramipril, dramatically reduced progression.[99] In the large recent Heart Protection Study (HPS), more than 20,000 subjects were randomised to receive either placebo or a combination of vitamin E, vitamin C or beta carotene for five years.[101] This combination of antioxidant vitamins had no effect on major coronary events, major vascular events, death or strokes. Likewise, treatment with a similar combination failed to prevent progression of coronary artery lesion development detected by quantitative coronary angiography, and in fact this combination seemed to negate the benefit of lipid-lowering strategies concomitantly administered in this study.[101]

On the surface, these studies would seem to

simply refute a role of ROS in vascular disease. We suggest, however, that these studies indicate that the commonly-used antioxidant vitamins are ineffective, but do not discount a role of oxidant stress in vascular disease. There are several important issues related to this direction of research that need to be considered in the design of future trials and also therapeutic strategies to reduce oxidative stress.

First, it should be emphasised that treatment with antioxidant vitamins may not effectively increase antioxidant levels within the vessel. As emphasised by Jiang *et al.*,[102] treatment with α-tocopherol, most commonly used in these trials, is associated with a marked decline in tissue levels of γ-tocopherol, such that the total tocopherol level may not be altered by such therapy. This may have serious pro-oxidant consequences, because γ-tocopherol is more effective in trapping lipophilic electrophiles than is α-tocopherol. A diet rich in antioxidants, vitamin-containing fruits and vegetables contains all forms of tocopherol. This might, in part, explain differences in the retrospective cohort studies (where dietary intake was considered) versus the prospective randomised trials (which employed α-tocopherol supplements) described above.

A second issue that is not commonly understood is that the reaction product between vitamin E or vitamin C with $O_2^{\bullet-}$ is H_2O_2. This is important, because it is likely that H_2O_2 is more pro-atherogenic than $O_2^{\bullet-}$. For example, a recent study by Tribble *et al.*[103] demonstrated that overexpression of SOD in transgenic mice had no effect on the development of atherosclerosis caused by cholesterol feeding. In fact, the severity of atherosclerosis in this study was linearly related to SOD activity, such that the greater the SOD activity, the worse the atherosclerosis. Thus, while $O_2^{\bullet-}$ may contribute to hypertension, and scavenging of $O_2^{\bullet-}$ with SOD or antioxidants may lower BP, these same interventions may have no effect or may actually promote atherosclerosis. Therapeutic approaches to reduce both $O_2^{\bullet-}$ and H_2O_2 formation would be highly preferable to simply enhancing the formation of H_2O_2 from $O_2^{\bullet-}$.

A third issue related to treatment approaches to correct oxidative stress is that we currently have no marker to allow one to determine who needs to be treated and to monitor the effectiveness of antioxidant therapy. This is akin to treating hypertension without measuring BP or treating hypercholesterolaemia without measuring plasma lipids. There are several promising markers, including urinary and plasma isoprostanes, the plasma ratio of oxidised to reduced glutathione, markers of DNA oxidation and markers of plasma lipid oxidation. Many of these require sophisticated methods for detection, such as HPLC-mass spectroscopy, that do not lend themselves to widespread clinical use. To date, none of these markers has been used in large trials. Nevertheless, a measurement of oxidative stress would prove extremely useful in allowing one to guide therapy in the future.

Inhibitors of the NAD(P)H oxidase and other antioxidants

There is substantial interest in developing specific inhibitors of the NAD(P)H oxidase. One such agent is apocynin (4-hydroxy-3-methoxyacetophenone), an ortho-methoxy-substituted catechol isolated from the traditional medicinal plant Picrorhiza kurroa.[104] Apocynin seems to block assembly of the phagocytic NAD(P)H oxidase, and has been used in a variety of animal models of inflammation, including arthritis, septic shock and asthma. Efforts have been made to increase apocynin's inhibitory activity by modifying its structure, and a methoxy substitution at the C5 position seems promising. Whether apocynin will be useful in humans remains uncertain. Another agent, aminoethyl benzenesulphono fluoride (AEBSF), also seems to inhibit assembly of the NAD(P)H oxidase, probably by altering the ability of the cytochrome b558 to bind $p47^{phox}$.[105] This agent is an irreversible serine protease inhibitor, and it is unlikely that its effects are specific for the oxidase. A recently discovered benzo(b)pyran-4-one derivative, S17834 has also been shown to inhibit NAD(P)H oxidase activity in cultured endothelial cells and to block TNF-stimulated VCAM-1 expression in these cells.[106] The specificity of this agent has not been demonstrated and it has not gained widespread use. Diphenylene iodinium (DPI) is widely used as an NAD(P)H oxidase inhibitor for *in vitro* studies; however, this agent affects activity of all flavin-containing proteins, and is not at all specific for NAD(P)H oxidases. In particular, it potently inhibits NO• synthesis by the NOSs.[107] A major challenge to developing inhibitors of the NAD(P)H oxidase is the need to target the vascular oxidases, while not affecting the oxidase of phagocytic cells. Non-specific inhibitors would almost certainly impair immune responses, leading to a condition similar to chronic granulomatous disease.

Significantly, at least two currently employed treatment strategies seem very effective in lowering activity of the NAD(P)H oxidases and thus preventing formation of $O_2^{\bullet-}$ and H_2O_2. These include ACE inhibitors and angiotensin receptor blockers (ARBs). In studies of experimental animals, we have found that, even in control animals, treatment with ARBs reduces activity of the oxidases and completely prevents the increase in NAD(P)H oxidase activity caused by Ang II or atherosclerosis.[73,108]

Another commonly used therapy that inhibits NAD(P)H oxidase activity is with the HMG-CoA reductase inhibitors (statins). These agents prevent the production of geranylgeranyl di-phosphate, which is necessary for membrane association of Rac-1.[109,110] Because Rac-1 membrane translocation is important for full function of the oxidase, the statins have unexpected antioxidant effects. There is substantial interest in the so-called 'pleiotropic' effects of the statins, and the ability of these agents to reduce vascular production of ROS is almost certainly responsible for some of these non-lipid lowering effects.

A less specific approach to preventing vascular oxidant stress is to develop newer antioxidant compounds that are more potent than the traditionally employed antioxidant vitamins. One such compound, is butanedoic acid, [mono[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenyl] ester], or AGI 1067. This agent is a modified form of Probucol and, unlike the parent probucol, is water-soluble and does not prolong the QT interval. AGI 1067 has very potent antioxidant properties and has proven effective in reducing restenosis following stent placement in humans.[111] A large-scale clinical trial is currently underway to examine the ability of this agent to reduce cardiovascular events in patients with known atherosclerosis.

Concluding remarks

It is clear that the NAD(P)H oxidase and its activation by Ang II play a critical role in vascular disease. Our understanding of the vascular oxidases has grown substantially during the past five years; however, there remains a great deal to be learned about how the enzyme actually works, how the subcellular location of the oxidases affects local cellular redox state, what stimuli other than Ang II activate the oxidase, and what can be done to inhibit its activity. A concept that has arisen during the past several years is that ROS produced by the NAD(P)H oxidases can lead to additional oxidant stress by a variety of mechanisms, including oxidation of tetrahydrobiopterin and promotion of $O_2^{\bullet-}$ production by the NOSs,[79] depleting small molecule antioxidants and by oxidative inactivation of the Cu/Zn-containing SODs.[81] The pro-inflammatory properties of oxidant stress promote the entry of macrophages into the vessel wall, and these cells are capable of producing very large concentrations of ROS. There are almost certainly other downstream targets of oxidants produced by the NAD(P)H oxidases about which we must learn. Overall, this area of research continues to be very fruitful and a promising direction for future therapeutic intervention.

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