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NADPH Oxidases and Angiotensin II Receptor Signaling

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Abstract

Over the last decade many studies have demonstrated the importance of reactive oxygen species (ROS) production by NADPH oxidases in angiotensin II (Ang II) signaling, as well as a role for ROS in the development of different diseases in which Ang II is a central component. In this review, we summarize the mechanism of activation of NADPH oxidases by Ang II and describe the molecular targets of ROS in Ang II signaling in the vasculature, kidney and brain. We also discuss the effects of genetic manipulation of NADPH oxidase function on the physiology and pathophysiology of the renin angiotensin system.

Keywords

Angiotensin II; NADPH oxidases; reactive oxygen species; vascular; kidney; brain

Over a decade ago, it was observed that angiotensin II (Ang II) is able to activate a NADPH oxidase (Griendling et al., 1994) that mediates the hypertensive response induced by Ang II infusion (Rajagopalan et al., 1996). Since then, numerous studies have demonstrated that NADPH oxidases regulate several Ang II functions in different tissues, many of which contribute to hypertension. In this review, we will describe the consequences of Ang II-mediated NADPH oxidase activation, with a focus on the cardiovascular, renal and central nervous systems.

NADPH Oxidases

Structure and activity

All types of cells have the ability to produce reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and peroxynitrite. Among ROS, H_2O_2 is the most stable and has been most often implicated as a second messenger. It is mainly derived from dismutation of $O_2^{\cdot-}$ by superoxide dismutase (SOD), which in turn can be produced by multiple enzyme systems, of which NADPH oxidases are major sources in non-phagocytic cells.

The classical NADPH oxidase is a multimeric enzyme that originally was described in neutrophils, where it plays an antimicrobial role. The phagocytic NADPH oxidase contains a heterodimeric membrane-bound cytochrome b558 complex, which consists of gp91phox (renamed Nox2) and p22phox, and 3 cytosolic subunits (p47phox, p67phox and the small G-

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protein Rac). Upon agonist stimulation, these cytosolic subunits translocate to the cytochrome complex, leading to an increase in enzymatic activity. In humans, there are five different NADPH oxidase homologues called Nox1 to Nox5, and two related oxidases, Duox1 and Duox2, as well as two additional homologues of the cytosolic units, NoxO1 and NoxA1 (Banfi et al., 2003; Geiszt et al., 2003; Takeya et al., 2003; Ueyama et al., 2007). Although all Nox enzymes are able to increase intracellular ROS, there are important differences regarding their activation, subunit composition, localization and expression (Figure 1). For more details please refer to the following comprehensive reviews (Bedard and Krause, 2007; Hordijk, 2006; Sumimoto, 2008). Ang II has been functionally linked to Nox1 (Dikalova et al., 2005; Lassegue et al., 2001; Matsuno et al., 2005), Nox2 (Bendall et al., 2002; Lavigne et al., 2001; Pagano et al., 1997) and variably to Nox4 (Wingler et al., 2001) in the vasculature, Nox2 and Nox4 in the kidney (Block et al., 2008; Gorin et al., 2003; Haque and Majid, 2004) and Nox2 in the brain (Wang et al., 2006).

ROS derived from NADPH oxidases serve a signaling function by inducing specific biochemical changes in their molecular targets. H_2O_2 can oxidize the thiol group of protein (P) cysteines to sulfenic acid (P-SOH), disulfide (PSSP), sulfinic acid (P-SO₂H), or sulfonic acid (P-SO₃H), the latter of which is an irreversible state of thiol oxidation (Monteiro et al., 2008) (Figure 2). Thiol oxidation by H_2O_2 (Juarez et al., 2008) has been shown to inhibit protein tyrosine phosphatase (PTP) activity (Kwon et al., 2004; Mahadev et al., 2004; Tabet et al., 2008). Sensitivity to H_2O_2 is dictated largely by the pKa of the cysteines in the active site of these enzymes: they have an unusually low pKa, which renders them more susceptible to oxidation (Salmeen and Barford, 2005; Tonks, 2005). In contrast, superoxide reacts with iron-sulfur (Fe-S) centers of heme-containing molecules, resulting in altered activity. Two examples are inactivation of aconitase, leading to inhibition of mitochondrial function (Gardner et al., 1994), and activation of guanylate cyclase (Burke-Wolin et al., 1991; Burke and Wolin, 1987).

NADPH oxidase activation by Ang II

The mechanism of NADPH oxidase activation by Ang II is complex and still incompletely understood. It has been shown that Ang II-mediated ROS production in vascular smooth muscle cells (VSMCs) from large arteries is mediated by Nox1 activation, as shown by loss of the ROS response after treatment with an adenovirus expressing antisense Nox1 (Lassegue et al., 2001), while Nox2 appears to be the Ang II-responsive Nox in resistance vessels, as demonstrated in human VSMCs transfected with gp91phox antisense oligonucleotides (Touyz et al., 2002), in the heart, as shown using small interference RNA (siRNA) against Nox2 (Hingtgen et al., 2006) and in the kidney, as described in gp91phox knockout mice (Haque and Majid, 2004). Typically, there are two different phases of ROS production: one is rapid and transient and the other is delayed and sustained. The first peak is due to an acute activation of NADPH oxidases by Ang II, while the second is dependent on the initial burst and is a consequence of upregulation of different NADPH oxidase subunits by Ang II. For example, in VSMC, the sustained phase of ROS production is preceded by upregulation of Nox1 mRNA (Lassegue et al., 2001; Wingler et al., 2001), p22phox mRNA (Fukui et al., 1997)(Fukui et al., 1997) and protein (Touyz et al., 2002) and p47phox protein (Ni et al., 2007; Touyz et al., 2002). There is also one report that Ang II can upregulate Nox4 mRNA in the vasculature (Wingler et al., 2001), although this is not observed universally (Mollnau et al., 2002).

In VSMCs, rapid ROS release requires p47phox via its phosphorylation by protein kinase C (PKC) and subsequent translocation to the membrane (Lavigne et al., 2001; Seshiah et al., 2002; Touyz et al., 2002), where it organizes the activating subunits NoxA1 and Rac (Ambasta et al., 2006; Hassanain et al., 2007). While each of these subunits has been implicated in the

response to Ang II, actual formation of this complex in VSMCs remains to be shown biochemically. Ang II can stimulate PKC-dependent NADPH oxidase activity through three different phospholipases (PL): PLC (Karathanassis et al., 2002), PLD (Touyz and Schiffrin, 1999), and PLA₂ (Zafari et al., 1999), with PLD likely predominating. Another important mediator of p47phox phosphorylation is Src (Touyz et al., 2003), although it is not clear whether it regulates p47phox directly or by its actions on the cytoskeleton through cortactin, a Src substrate that binds p47phox (Agerer et al., 2005; Sangrar et al., 2007; Touyz et al., 2005) and facilitates the proper assembly and function of NADPH oxidases by Ang II (Touyz et al., 2005).

Ang II activation of Nox1 and Nox2 also requires Rac1 (Cheng et al., 2006; Miyano et al., 2006; Ueyama et al., 2006). Stimulation of Rac1 by Ang II seems to be mediated by activation of the guanine nucleotide exchange factor (GEF), SOS-1 (Zuo et al., 2005), although a relationship between SOS-1 and Nox activation remains to be demonstrated. Activation of the Rac-GEF is dependent upon Src-mediated transactivation of the epidermal growth factor receptor (EGFR), which then serves as a binding site for phosphatidylinositol 3-kinase (PI-3K), an immediate activator of the Rac-GEF (Seshiah et al., 2002). This initial activation step appears to occur in caveolae (Ushio-Fukai et al., 2001), the site of Nox1 localization (Hilenski et al., 2004). Of importance, the H₂O₂ that is produced as a result of initial NADPH oxidase activation then activates Src, creating an amplification of oxidase activity (Seshiah et al., 2002). Prolonged signal generation requires upregulation of the Nox subunit, p47phox and p22phox (Fukui et al., 1997; Lassegue et al., 2001; Touyz et al., 2002).

ANG II Activation of NADPH Oxidases in the Cardiovascular System

Hypertension

Ang II is a major contributor to hypertension via its central, vascular and renal effects. It is clear that NADPH oxidase-derived ROS production plays a critical role in the hypertension induced by Ang II (Dikalova et al., 2005; Fukui et al., 1997; Landmesser et al., 2002; Rajagopalan et al., 1996; Weber et al., 2005). For example, our group demonstrated that p22phox is increased in hypertensive rats (Fukui et al., 1997), that smooth-muscle specific p22phox overexpressing mice have elevated blood pressure (Weber et al., 2005), and that smooth-muscle specific overexpression of Nox1 in mice induces a higher pressure response to Ang II (Dikalova et al., 2005). It should be noted that overexpression of one subunit is often accompanied by an increase in the others, and in most reported cases results in an increase in NADPH oxidase activity (Dikalova et al., 2005; Fukui et al., 1997; Laude et al., 2005; Weber et al., 2005). For example, overexpression of p22phox increases the expression of Nox1 protein and NADPH oxidase activity (Laude et al., 2005). In contrast, in p47phox- or Nox1-deficient mice, the Ang II effects on vascular tone and blood pressure are inhibited (Gavazzi et al., 2006; Landmesser et al., 2002; Matsuno et al., 2005). The infusion of Ang II does, however, induce hypertension in Nox2 deficient mice (Touyz et al., 2005; Wang et al., 2001). Other studies showed that different ROS scavengers, including catalase (Wilson, 1990; Yang et al., 2003) and SOD (Gongora et al., 2006; Wilson, 1990), blunt the response to Ang II. In seeming contrast to these results, a recent study demonstrated that in a model of chronic Ang II elevation, deletion of Nox1 has no effect on blood pressure, but does improve hypertension-related end-organ damage (Yogi et al., 2008). Similarly, Liu et al. (Liu et al., 2003) infusion of the Nox2 inhibitor gp91-dstat into rats co-infused with AngII for 1 week attenuated the increase in medial hypertrophy, but not blood pressure. It is thus possible that NADPH oxidases are necessary for the development, but not the maintenance, of hypertension by Ang II, and contribute to its pathophysiological effects.

With respect to the role of H₂O₂ and O₂⁻ in the regulation of vascular tone per se, the observed response is tissue and concentration dependent (Barlow and White, 1998; Wei et al., 1996;

Yang et al., 1999; Yang et al., 1998). For example, $O_2^{\cdot-}$ and H_2O_2 have vasodilator effects in cerebral arteries, while they have a vasoconstrictor effect in non-cerebral arteries (for more details, please refer to (Miller et al., 2006)). Superoxide exerts its effects mainly, but not exclusively, by inactivating nitric oxide, while H_2O_2 acts via upregulation of endothelial nitric oxide synthase (eNOS) (Drummond et al., 2000) and via direct effects on ion channels (Liu and Gutterman, 2002) or the contractile machinery (Jin and Rhoades, 1997). In coronary vessels, H_2O_2 is a potent vasodilator acting via modulation of K^+ channels (Miura et al., 2003; Sato et al., 2003). The ROS produced by Ang II activation of NADPH oxidases thus modulate vascular tone by regulating both endothelial function and VSMC contraction.

Endothelial cells

Endothelial dysfunction is largely thought to be a consequence of hypertension, although recent studies suggest that endothelial dysfunction also may predispose to the development of hypertension (Schlaich et al., 2004; Taddei et al., 1996; Yang et al., 2007). Impaired endothelium-dependent relaxation results from a decrease of nitric oxide bioavailability, largely due to increased $O_2^{\cdot-}$. The link between Ang II, ROS and endothelial dysfunction had been demonstrated in hypertensive animals. Acute infusion of Ang II results in impaired relaxation to acetylcholine, the calcium ionophore A23187, and nitroglycerin (Rajagopalan et al., 1996). Moreover, Ang II, through PKC activation, induces an upregulation of Nox1 (Lassegue et al., 2001) and uncouples eNOS (Mollnau et al., 2002), thus increasing $O_2^{\cdot-}$ production in a positive feedback fashion. Interestingly, these effects are accompanied by a decrease in soluble guanylate cyclase and a diminished activation of protein kinase G (Mollnau et al., 2002). In other models of hypertension such as the two kidney-one clip (2K-1C) model, it has been demonstrated that Ang II-induced NADPH oxidase activation is responsible for impaired vasodilator responses to endogenous and exogenous nitrovasodilators (Heitzer et al., 1999). Recently, Doughan et al. (Doughan et al., 2008) demonstrated that Ang II activation of NADPH oxidases leads to mitochondrial dysfunction in endothelial cells, amplifying the increase in ROS. This represents a new pathophysiological pathway of Ang II activation.

Vascular smooth muscle cells

Contraction—Ang II is one of the most powerful vasoconstrictor agents for vascular smooth muscle. As with other vasoconstrictors, phosphorylation of the myosin light chain (MLC) is a crucial step in force development to Ang II. The rate of MLC phosphorylation depends on a balance between Ca^{2+} /calmodulin-dependent MLC kinase and myosin phosphatase activity (Somlyo and Somlyo, 2000). Thus, an increase in MLC kinase stimulation or a decrease in MLC phosphatase activity leads to VSMC contraction. ROS have been implicated in both of these pathways (Figure 3).

One of the most important early signals in the initiation of contraction by Ang II is thus a PLC-mediated increase in intracellular calcium concentration ($[Ca]_i$). Torrecillas et al. (Torrecillas et al., 2001) demonstrated that H_2O_2 production by Ang II in primary aortic VSMCs (Rodriguez-Puyol et al., 2002) mediates this increase in intracellular calcium concentration ($[Ca]_i$), and therefore MLC phosphorylation, without modifying inositol 1,4,5-trisphosphate (IP_3) production by PLC. This suggests that ROS production by Ang II may modify the affinity of the IP_3 receptor for its ligand. In support of this idea, Redondo et al. (Redondo et al., 2004) observed that the H_2O_2 -induced increase in $[Ca]_i$ in platelets is abolished by preincubation with Xetosphonin C (an IP_3 receptor inhibitor). However, other studies have shown that activation of Src (Touyz et al., 2001) and ERK (Touyz et al., 1999) by Ang II is upstream of IP_3 production and the increase in $[Ca]_i$, suggesting that they may be the actual targets of H_2O_2 because they have previously been shown to be redox sensitive (Touyz et al., 2003; Ushio-Fukai et al., 2001; Viedt et al., 2000). Because the activation of ERK by ROS is not universally observed (Pinzar et al., 2005; Sano et al., 2001; Touyz et al., 2003; Viedt et al.,

2000) and Src activation is only partially ROS dependent (Seshiah et al., 2002), it is likely that ROS generation by Ang II may also be necessary for the correct release of calcium by IP₃. In fact, [Ca]_i is enhanced in arteries from spontaneously hypertensive rats, and this increase is both ROS- and Ang II-dependent (Tabet et al., 2004). Another mechanism responsible for the ROS-sensitivity of the Ang II-induced increase in [Ca]_i is calcium channel activation. Thus, it has been shown that increased expression of the α_1C subunit of the L-type calcium channel by Ang II is ROS-, PKC- and CREB-dependent (Tsai et al., 2007), and the function of this channel is regulated by NADPH oxidases (Wang et al., 2006). Finally, it has been demonstrated that H₂O₂ inhibits sarcoendoplasmic reticulum Ca-ATPase (SERCA), thereby blocking the reuptake of calcium from the cytosol to the internal stores and increasing [Ca]_i. Inhibition of SERCA occurs through oxidation of specific sulphhydryl groups (Redondo et al., 2004), although the source of the responsible ROS is unclear. Thus, it is likely that ROS regulate the increase in [Ca]_i induced by Ang II at multiple levels.

As noted above, the other factor that regulates the levels of phosphorylated and therefore activated MLC is the activity of MLC phosphatase, which is inhibited by RhoA/RhoA kinase (ROCK) (Seko et al., 2003)-mediated phosphorylation on Thr696 of MYPT1, a myosin binding subunit of MLC phosphatase. Importantly, Uehata et al. (Uehata et al., 1997) observed that ROCK is implicated in the pathophysiology of hypertension induced by Ang II. Recently, it has been shown that the Ang II-stimulated ROCK-mediated phosphorylation of MLC (Seko et al., 2003) is dependent on NADPH oxidases (Higashi et al., 2003). The mechanism of activation of RhoA/ROCK by ROS is not clear, but may involve PDZ- (Hilgers et al., 2007) and leukemia-associated (Ying et al., 2006) GEFs. More studies are necessary to define the actual molecular target of ROS in this pathway.

Hypertrophy—Many of the pathophysiological effects of Ang II are due to its pro-growth properties. In vivo proof that these events are ROS-dependent comes from studies in genetically modified animals. Thus, overexpression of Nox1 (Dikalova et al., 2005) or p22phox (Weber et al., 2005) potentiates the effect of Ang II on vascular hypertrophy, while knockout of Nox1 (Matsuno et al., 2005) or Nox2 (Bendall et al., 2002) inhibits it. Similarly, Ang II-induced cardiac hypertrophy requires the Nox activator Rac1 (Satoh et al., 2006). In both VSMCs and cardiomyocytes, multiple molecular targets that are activated by Ang II in a ROS-dependent manner contribute to hypertrophy, including tyrosine kinases, mitogen activated protein kinases (MAPK), the survival kinase Akt and redox-sensitive transcriptional factors (Taniyama et al., 2004; Touyz et al., 2003; Touyz et al., 2004; Ushio-Fukai et al., 1999) (Figure 4). In addition, ROS-mediated inactivation of protein tyrosine phosphatases (PTP) amplifies growth-related signaling. Classic PTPs include transmembrane receptor-like proteins (RTPs) and non-transmembrane cytoplasmic PTPs. All members of the PTP family are characterized by the presence of a highly conserved sequence HCXXGXXRS/T, which contains a Cys residue that is necessary for catalysis. As mentioned earlier, the thiol oxidation in this Cys by addition of H₂O₂ leads to the inactivation of the PTP activity (Juarez et al., 2008). A recent study has shown that SOD1 is essential for PTP inactivation by growth factors, indicating that H₂O₂, but not O₂⁻, is implicated in PTP inactivation (Juarez et al., 2008). Tabet et al. (Tabet et al., 2008) demonstrated that Ang II oxidizes PTP/SHP-2 via Nox1 activation, and that PTP/SHP-2 inactivation regulates Ang II-induced activation of Akt, but not of ERK1/2 or p38 MAPK. Other studies have demonstrated that H₂O₂ inactivates phosphatase and tensin homolog (PTEN) (Lee et al., 2002; Leslie et al., 2003), thus increasing the concentration of PIP₃ and activating Akt (Leslie et al., 2003), low molecular weight PTP (LMW-PTP) (Caselli et al., 1998), and Cdc25 (Savitsky and Finkel, 2002). However, whether Ang II similarly inactivates these phosphatases remains to be determined.

One of the early effects of Ang II stimulation is c-Src activation, which is inhibited by catalase overexpression or treatment with pharmacological antioxidants (Ushio-Fukai et al., 2001).

Phosphorylated Src mediates the recruitment of the AT₁R to caveolae, which is required for proper organization of ROS-dependent Ang II signaling. Ang II also transactivates the IGF-1R (Touyz et al., 2003) and PDGFR via ROS and Shc (Heeneman et al., 2000). Transactivation of these receptors leads to the recruitment and stimulation of different proteins, including phosphatidylinositol 3-kinase, which activates Akt (Ushio-Fukai et al., 1999). Whether the ROS sensitivity of this pathway is conferred by Src, or if there are redox modifications of these proteins or their associated phosphatases, remains to be determined. Because Akt is phosphorylated by MAPKAPK-2, a substrate of the ROS-sensitive p38MAPK (Taniyama et al., 2004), it is possible that it is p38MAPK activation that is the actual target of ROS. One intriguing hypothesis is that ROS mediate the dissociation of the p38MAPK kinase Ask-1 from redox-active thioredoxin (Hsieh and Papaconstantinou, 2006; Matsuzawa et al., 2005), although this remains to be investigated.

Activation of other MAPKs by Ang II also requires ROS derived from NADPH oxidases. Thus, c-Jun NH2 terminal kinase (JNK) activation in response to Ang II is blunted by different antioxidants (Kyaw et al., 2001) or by transfection with antisense against the p22phox subunit (Viedt et al., 2000). Moreover, Shc is implicated in the phosphorylation of JNK (Yoshizumi et al., 2001). In contrast to p38MAPK and JNK, whose redox sensitivity to Ang II is nearly universal in the vasculature, the sensitivity of ERK1/2 to ROS is controversial. Some studies found that activation of ERK1/2 is ROS independent (Kyaw et al., 2001; Touyz et al., 2003; Viedt et al., 2000), while others found it to be ROS sensitive (Frank et al., 2000; Pinzar et al., 2005; Sano et al., 2001). This suggests that ERK1/2 is not an actual target of ROS; rather, when redox-sensitivity is observed, it is likely conferred by an upstream kinase or phosphatase and may occur in a specific subcellular compartment.

A number of years ago, we suggested that some aspects of Ang II signaling may occur subsequent to internalization of the AT₁R in endosomes (Griendling et al., 1986). The apparent role of caveolae in organizing redox signals (Zuo et al., 2005) suggests that ROS-dependent signaling is intimately linked to vesicular trafficking. A recent series of elegant papers by the Englehardt and Miller labs shows that Nox-dependent ROS generation in response to IL-1 β or TNF- α occurs in the early endosome and is released into the cytosol by a pathway requiring activation of chloride channels (Miller et al., 2007; Mumbengegwi et al., 2008), although the origin of the vesicles is unknown. This provides a molecular explanation for the finding that ROS generation in nonphagocytic cells is intracellular, even though the Nox enzymes are oriented to the outside of the plasma membrane. Although this has not been studied for Ang II, it is likely that a similar mechanism explains the robust intracellular generation of H₂O₂ observed in VSMCs, and the apparent compartmentalization of redox signaling in these cells. Both caveolin-mediated and clathrin-dependent internalization have been shown for AT₁R internalization (Gaborik et al., 2001; Ishizaka et al., 1998), but have not been studied with respect to Ang II stimulated ROS generation. However, Choi et al. (Choi et al., 2008) demonstrated that β -arrestins are not necessary for Nox1 activation by Ang II, which may indicate that activation occurs prior to internalization. Because this latter study was conducted entirely in a reconstituted system, these results must be interpreted with caution.

Activation of these ROS-sensitive signal transduction pathways ultimately leads to stimulation of specific transcription factors. One of the most well-studied is AP-1, a heterodimer of Fos and Jun. Viedt et al. (Viedt et al., 2004) and Wu et al. (Wu et al., 2005) demonstrated that activation of a p22phox- and gp91phox-containing oxidase is essential for Ang II-stimulated AP-1 binding to DNA in VSMCs and cardiomyocytes. Conversely, activation of AP-1 is necessary for increased transcription of p22phox by Ang II, suggesting a feed-forward mechanism (Manea et al., 2008). AP-1 is implicated in diverse biological functions, such as cell proliferation, protein synthesis, apoptosis and secretion of pro-fibrotic factors via its induction of specific genes. With respect to Ang II, the increase in TGF- β , endothelin, MMP-1

and fibronectin mRNA expression is mediated by ROS- and AP-1 dependent mechanisms (Browatzki et al., 2005; Cheng et al., 2003; Moriguchi et al., 1999; Wenzel et al., 2001). Another important ROS-dependent transcription factor activated by Ang II is NF κ B. The prototype of the NF κ B family is the p50/p65 heterodimer expressed constitutively in most mammalian cells. It is well established that NF κ B is complexed with and sequestered in the cytoplasm by inhibitory I κ B (inhibitor of NF κ B) proteins, and that many activating stimuli induce phosphorylation of I κ B by the I κ B-kinase complex (IKK α , IKK β , and IKK γ), initiate I κ B ubiquitination and degradation by means of the 26S proteasome, and allow translocation of NF κ B to the nucleus (Chen et al., 1996; DiDonato et al., 1996; DiDonato et al., 1995; Ghosh and Karin, 2002). Although many investigators have shown that NF κ B is redox sensitive, there are just a few studies that demonstrated the activation of this factor by ROS in VSMCs (Browatzki et al., 2005; Ortego et al., 1999; Shang et al., 2008). Among the different targets regulated by Ang II in a NF κ B dependent manner is monocyte chemoattractant protein-1 (MCP-1) (Capers et al., 1997; Chen et al., 1998). Other Ang II- and ROS-sensitive transcription factors are cyclic AMP response element-binding protein (CREB) (Funakoshi et al., 2002; Ichiki et al., 2003; Tsai et al., 2007), hypoxia inducible factor 1- α (Gorlach et al., 2001), Id3 and GKLf (Nickenig et al., 2002). These latter two factors have opposite effects on cell cycle progression, suggesting that ROS must be compartmentalized. Recently, it has been shown that Ang II-induced activation of Ets-1, which is important in vascular remodeling (Zhan et al., 2005), is redox sensitive and its activation induces an increase in p47phox expression (Ni et al., 2007). Ets-1 is also an important regulator of the cyclin-dependent kinase inhibitor p21^{CIP} (Zhang et al., 2003), plasminogen activator inhibitor-1 (PAI-1) and MCP-1 (Zhan et al., 2005).

ANG II Activation of NADPH Oxidases in the Kidney

The kidney also expresses NADPH oxidases, the most abundant of which is Nox4 (originally called Renal NADPH oxidase or Renox) (Geiszt et al., 2000). The effect of Ang II on NADPH oxidase activity in this organ is well described, although the molecular mechanisms are less clear and have been investigated mainly at the level of gene expression. Thus, infusion of Ang II upregulates the expression of p22phox and Nox1 and downregulates the expression of extracellular superoxide dismutase (EC-SOD) (Chabrashvili et al., 2003). The decrease of EC-SOD seems to participate in the upregulation of p22phox and Nox1. Moreover, the resulting increase in ROS production by Ang II in the kidney is important in the regulation of renal function (Chabrashvili et al., 2003; Jaimes et al., 2005; Lopez et al., 2003; Modlinger et al., 2006; Nouri et al., 2007). Thus, López et al. (Lopez et al., 2003) observed that renal infusion of Ang II in rats reduced renal blood flow (RBF), glomerular filtration (GF), sodium excretion, and NO levels, all of which were blunted by the AT₁R antagonist valsartan, the superoxide scavenger tempol and the NADPH oxidase inhibitor apocynin. Furthermore, treatment of rats with siRNA against p22phox prevents the increase of renal cortical NADPH oxidase activity and increases the excretion of the lipid peroxidation marker 8-isoprostane PGF_{2 α} induced by perfusion with Ang II (Modlinger et al., 2006). The role of superoxide in regulating renal function during Ang II infusion has been confirmed in a number of studies (Kopkan et al., 2006; Mori and Cowley, 2003; Mori et al., 2007; Wang et al., 2003; Welch et al., 2005).

Similar to its effects in VSMCs, Ang II can stimulate protein synthesis in mesangial cells (MC) and tubular cells (Hannken et al., 1998; Jaimes et al., 1998), leading to hypertrophy (Figure 4) and synthesis of extracellular components. Both processes contribute to the pathogenesis of fibrosis of the glomerular microvascular bed (Ardaillou et al., 1999). However, whereas in VSMC Ang II activation of the NADPH oxidase has been studied extensively in renal cells, it is less well understood. Similar to VSMC, the activation of the NADPH oxidase is dependent on the production of arachidonic acid (Block et al., 2006; Gorin et al., 2001), PKC (Jaimes et al., 1998) and Rac1 (Gorin et al., 2003); however, the principal Nox that is coupled to Ang II

is Nox4 (Gorin et al., 2003). Because Nox4 has been demonstrated to be Rac1 independent (Martyn et al., 2006), the significance of Rac1 in this system requires further study. As in VSMC, multiple targets are downstream of Nox activation by Ang II, including Akt (Gorin et al., 2003), EGFR (Ding et al., 2007), JNK (Ding et al., 2007), ERK1/2 (Yu et al., 2005) (Gorin et al., 2004), ETS-1 (Pearse et al., 2008), cyclooxygenase-2 (COX2) (Jaimes et al., 2005; Jaimes et al., 2008; Pearse et al., 2008) and PDK1 (Block et al., 2008). Some of the extracellular components regulated by ROS in Ang II-stimulated MC include fibronectin (Block et al., 2006; Pearse et al., 2008), collagen I (Zhao et al., 2008) and TGF- β (Lodha et al., 2002; Zhao et al., 2008), a profibrotic cytokine.

ANG II Activation of NADPH Oxidases in the Central Nervous System

Ang II in the brain participates in regulation of blood pressure, sympathetic activity, vasopressin release, thirst, and sodium appetite (Phillips, 1987). In the last several years, numerous studies have demonstrated that ROS generation by Ang II modulates some of these effects in the CNS, acting in different regions of the brain. Zimmerman et al. observed that the acute intracerebroventricular injection (Zimmerman et al., 2002) or chronic systemic perfusion (Zimmerman et al., 2004) of Ang II induced $O_2^{\cdot-}$ production in the subfornical organ (SFO), leading to an increase in blood pressure and dipsogenesis. Notably, transfection with adenovirus that expresses Mn-SOD or Cu/Zn SOD blocked these effects of Ang II. Moreover, studies from Zucker's group (Gao et al., 2004) demonstrated that the Ang II-NADPH oxidase axis is regulated by sympathetic excitation in the rostral ventrolateral medulla (RVLM), which is implicated in chronic heart failure (Gao et al., 2005). Ang II increases gp91phox and p47phox expression in the RVLM as well (Gao et al., 2004).

The mechanism of NADPH activation by Ang II in the CNS is incompletely understood. Even so, it has been demonstrated that NADPH oxidase activation requires Rac1 (Zimmerman et al., 2004) and PKC (Wang et al., 2006). More information is available concerning the downstream consequences of NADPH oxidase-derived ROS in the CNS (Figure 5). Thus, Wang et al. (Wang et al., 2006) observed that inhibition of NADPH oxidases by apocynin or an inhibitory Nox2 peptide docking sequence decreased Ang II-stimulated increases in $[Ca]_i$, and, more recently, it was observed that $O_2^{\cdot-}$ production by Ang II is implicated in the elevation of $[Ca]_i$ (Zimmerman et al., 2005) in neuroblastoma Neuro 2A cells. In addition, ROS production by Ang II has been shown to be critical for the inhibition of the delayed rectifier potassium current ($I(K_v)$), which contributes to neuronal activity (Sun et al., 2005). Other targets of Ang II stimulated NADPH oxidases in the CNS are p38 and ERK1/2. Chan et al. (Chan et al., 2005) demonstrated that Ang II injection in the RVLM increased the phosphorylation of ERK1/2 and p38MAPK, but not in JNK. These effects were attenuated by application into the RVLM of a flavin oxidase inhibitor, DPI, antisense oligonucleotides that target p22phox or p47phox mRNA, or Tempol. Moreover, they observed that DPI, Tempol and the p38MAPK inhibitor decreased the frequency of glutamate-sensitive spontaneous excitatory postsynaptic currents and the effects on the blood pressure induced by Ang II (Chan et al., 2005).

Conclusions and Future Directions

Multiple studies have demonstrated a role for NADPH oxidases in Ang II function. Although many downstream targets have been identified, the temporal sequence of events that lead to the proper activation and function of NADPH oxidase is not completely understood, and the compartmentalization of signaling remains to be fully investigated. In addition, more work is necessary to identify the precise molecular modifications of the putative signaling targets of ROS after Ang II stimulation, with particular attention to phosphatases. Understanding which NADPH oxidases are activated by Ang II in specific tissues, in normal physiology and in

disease development is also important, and may help us to define better interventions aimed at blocking the deleterious effects of Ang II activation while supporting those necessary for survival.

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Figure 1. Structure of the different vascular NADPH oxidases
 Different homologues of NADPH oxidases have been found in the vasculature. NoxA1 (NADPH oxidase activator 1) and NoxO1 (NADPH oxidase organizer 1).

Figure 2. Inactivation of protein tyrosine phosphatases (PTP) by H₂O₂-induced thiol oxidation of cysteine residues

H₂O₂ can inactivate PTPs either irreversibly (right side) or reversibly (left side). Reactivation of the PTPs requires reduction via thioredoxin (Trx) or glutathione (GSH).

Figure 3. Role of ROS in contraction induced by angiotensin II in vascular smooth muscle cells
 ROS production is implicated in calcium dependent and independent induced by angiotensin II. Red lines show inhibitory effects and blue lines depict activation. ROS (reactive oxygen species), AT₁R (angiotensin II receptor type 1), PLC (phospholipase C), PIP₂ (phosphoinositol biphosphate), IP₃ (inositol 1,4,5 triphosphate), IP₃R (inositol 1,4,5 triphosphate receptor), SERCA (sarco/endoplasmic reticulum Ca²⁺-ATPase), GEF (guanine nucleotide exchange factor), GTP (guanosin triphosphate), ROCK (Rho-associate kinase) and MLC (myosin light chain).

Fig 4. Role of ROS in the hypertrophy induced by angiotensin II in vascular smooth muscle cells (left panel) or mesangial cells (right panel)

Vascular smooth muscle and mesangial cells exhibit differences in the targets activated in the hypertrophy induced by angiotensin II. ROS (reactive oxygen species), AT₁R (angiotensin II receptor type 1), PKC (protein kinase C), PLD (phospholipase D), PLA₂ (phospholipase A₂), EGFR (epidermal growth factor receptor), PDGFR (platelet derived growth factor receptor), IGFR (insulin like growth factor receptor) PI₃K (phosphatidylinositol 3-kinase), PDK1 (phosphoinositide-dependent protein kinase 1), SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase), PTEN (phosphatase and tensin homolog), LMW-PTP (low molecular weight phosphatase), p38MAPK (p38 mitogen-activated protein kinase), MAPKAPK-2 (MAPK-activated protein kinase-2), JNK (C-Jun N-terminal kinases), ERK (extracellular signal-regulated kinases), AP-1 (activator protein 1), NF-κβ (nuclear factor-kappa β), CREB (cAMP response element-binding), Ets-1 (E26 transformation-specific sequence), GFLK (Gut-enriched Krüppel-like factor) and COX-2 (cyclooxygenase 2).

Fig 5. Role of ROS in modulation of angiotensin II signaling in the brain

ROS are implicated in the dipsogenic action and blood pressure modulated by angiotensin II in the brain. ROS (reactive oxygen species), AT1R (angiotensin II receptor type 1), PKC (protein kinase C), p38MAPK (p38 mitogen-activated protein kinase) and ERK (extracellular signal-regulated kinases).