Angiotensin II–Induced Reactive Oxygen Species and the Kidney

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The renin-angiotensin-aldosterone system (RAAS) plays a pivotal role in regulating physiologic and pathophysiologic processes in the kidney. Although different components of the RAAS, such as renin, aldosterone, and various angiotensin fragments, can initiate renal impairment on their own, angiotensin II (AngII) is the primary effector of this system. AngII was originally identified as a vasoconstrictor and potent stimulus of aldosterone release from the suprarenal glands and also has been implicated in the regulation of glomerular filtration and tubular transport. Intensive research in the past 15 yr has provided convincing evidence that AngII is a key contributor to progression of renal disease by stimulating growth, inflammation, and fibrosis of the kidney.

AngII binds to specific receptors to mediate its particular effects. The angiotensin type 1 (AT1) and type 2 (AT2) receptors are the best characterized receptors on a molecular level, but additional types may exist. Most of the known physiologic and pathophysiologic effects of AngII are transduced by the AT1 receptor, a 359-amino acid protein that belongs to the seven-membrane superfamily of G-protein–associated receptors. After the binding of AngII to the AT1 receptors, a series of signaling cascades is activated. Although traditionally divided into G-protein– and non–G-protein–related signaling, there are so many interactions between these subgroups of AngII-induced signaling pathways that a strict distinction becomes difficult. An example of a G-protein–dependent pathway is activation of phospholipase C with the subsequent production of inositol 1,4,5-phosphate and diacylglycerol. Non–G-protein pathways induced by AngII are phosphorylation and the activation of various tyrosine kinases. AngII is an important mediator of oxidative stress, and reactive oxygen species (ROS) induced by AngII are chief signal intermediates in several signal transduction pathways involved in renal pathophysiology. Moreover, AngII-induced ROS are important for renal growth processes, inflammation, and fibrosis. This brief review highlights how AngII stimulates ROS formation and how ROS contribute to kidney injury.

WHAT ARE ROS?

ROS are composed of a series of oxygen intermediates, including the free radical superoxide anion (\(\text{O}_2^-\)), the nonradical hydrogen peroxide (\(\text{H}_2\text{O}_2\)), the highly reactive hydroxyl free radical (\(\text{OH}\)), peroxynitrite (\(\text{ONOO}^-\)), and singlet oxygen (\(\text{O}_2^1\)), in which one of the electrons is...
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raised to an orbital of higher energy with an inversion of spin. Some of the pathways for generation and metabolism of ROS are shown in Figure 1. The original source is O2, which is univalently reduced to form O2·− by multiple enzymatic pathways (Figure 1). O2·− is unstable in aqueous solutions with a half-life of seconds.6–10 It is catalyzed into H2O2 by superoxide dismutase. This relatively weak oxidant holds a central position in the further metabolism to other ROS. H2O2 can oxidize chloride to form the reactive hypochlorous acid (HOCl) in the presence of myeloperoxidase (MPO). The highly reactive hydroxyl radical (·OH) can also be formed from H2O2. Alternatively, O2·− is detoxified by catalase to H2O.

In addition, there are several interactions between ROS and nitric oxide (NO) leading to a decrease in NO bioavailability.8,11 O2·− rapidly reacts with NO, yielding ONOO−, which may decompose into nitrate and ·OH. ONOO− also oxidizes the zinc-thiolate center of NO synthase (NOS), resulting in a decrease in NO formation.8 H2O2 reacts with the heme center of myeloperoxidase to produce Fe3+ that, in turn, oxidizes NO to NO2−.9,12 Another interaction between ROS and NO is the oxidation of the NOS co-factor tetrahydrobiopterin (BH4). In the absence of BH4, the NOS rather forms O2·− instead of NO, a process called NO uncoupling, leading to increased oxidative stress.13,14 Finally, ROS induce lipid and protein oxidation, generating various further active compounds such as lipid peroxyl (LOO·) and lipid alkoxyl radicals (LO·).15–17 Because of the highly reactive nature of ROS with the potential of deleterious effects on cell integrity, ROS must be neutralized by protective enzymes and endogenous antioxidants (Figure 1). Because H2O2 is less reactive than O2·−, superoxide dismutase may be considered as part of a detoxification pathway neutralizing superoxide anions.12 Furthermore, H2O2 is reduced by catalase or glutathione peroxidase to H2O. In particular, the tetrameric glutathione peroxidase serves as a detoxification pathway for several noxious lipid peroxides.

The key initial step in the formation of all ROS is the conversion of molecular O2 into O2·−. Several enzymatic pathways can generate O2·− (Figure 1), but in overall quantitative terms, it is the electron transport chain in mitochondria that is the most important source. For AngII-mediated ROS generation, the NAD(P)H oxidase is the central enzyme complex.11,12

**NAD(P)H OXIDASE COMPLEX**

The “classic” NAD(P)H oxidase is an enzymatic complex that is responsible for the generation of O2·− in neutrophils during the respiratory burst.13 Under normal conditions, the prototypic neutrophil NAD(P)H oxidase is dormant in nonactivated neutrophils with only two subunits, glycoprotein (gp)91phox (for phagocyte oxidase) and p22phox, comprising the membrane-bound cytochrome b558 (Figure 2). The flavoprotein FAD is a co-factor linking NADPH and cytochrome b558. Two isoforms of the small GTP-binding protein Rac, Rac1 and Rac2, promote the assembly of the NAD(P)H oxidase multienzyme complex and may act as a switch that triggers the electron transport.18 Rac2 exhibits a high affinity for cytochrome b558 and seems to be constitutively associated with the cell membrane. During the respiratory burst and cellular activation, the additional components p67phox, p47phox, p40phox, and Rac1 shift from the cytosol to the membrane (Figure 2). These proteins bind to the poly-L-lysine–rich domain of p22phox through the interaction of the src homology domain-3 (SH3). Furthermore, the SH3-mediated interaction induces the combination of p67phox with p47phox. The

![Figure 2. The multienzyme complex that constitutes neutrophil NAD(P)H oxidase. The subunits Rac1/2, p67phox, p47phox, and p40phox reside under normal conditions in the cytosol and associate with the membrane-bound Nox/p22phox subunits only after activation. p47phox interacts with components of the cytoskeleton in different cell types, different Nox subunits with p22phox. AngII stimulates transcription of different NAD(P)H oxidase subunits such as p22phox and p47phox or various Nox proteins. In addition, AngII stimulation results in activation of the enzyme complex by association of the subunits. The associated enzyme complex generates ·O2−.](image-url)
translocation activates the NAD(P)H oxidase to generate large amounts of \( \cdot \mathrm{O}_2^- \) (approximately 10 nmol/min per 10^6 cells) in the extracellular environment.\textsuperscript{18}

Although all components of the classic neutrophil NAD(P)H oxidase are also found in nonphagocytic cells (e.g., in endothelial cells), there are several important structural and functional differences.\textsuperscript{19,20} Nonphagocytic NAD(P)H oxidase enzymes continuously generate low levels of \( \cdot \mathrm{O}_2^- \) intracellularly and can be further stimulated by several agonists, including AngII. In fact, there is evidence that several of the subunits are fully preassembled intracellularly in a perinuclear distribution associated with the cytoskeleton.\textsuperscript{21} Moreover, at least five isoforms of gp91phox, named Nox1 through 5, have been characterized, whereas the classic neutrophil isoform gp91phox was renamed Nox2.\textsuperscript{18} Nox1 and 3 through 5 are expressed in nonphagocytic cells. In the kidney, all components of the neutrophil NAD(P)H oxidase including Nox2 are present in endothelial cells.\textsuperscript{19,20,23} In addition, Nox4 is widely expressed in renal cells, and p47phox is strongly expressed in glomeruli. In colon epithelial cells, homologues of p47phox and p67phox (NOXO 1 and NOX1A) are found, but an expression in renal cells has not yet been studied. There are also structural differences, and Nox5 does not require p22phox as a docking module, whereas Nox4 operates constitutively, not requiring cytosolic subunits.

**ANGII-INDUCED ROS FORMATION**

More than a decade ago, it was concluded from indirect evidence that AngII may induce ROS formation.\textsuperscript{24} Acute AngII infusion experiments into naive rats in the presence or absence of various free radical scavengers were performed, and these scavengers partly inhibited vascular hyperpermeability and cellular damage.\textsuperscript{22,23} Subsequent studies demonstrated that treatment of cultured vascular smooth muscle cells with AngII for 4 to 6 h increased \( \cdot \mathrm{O}_2^- \).\textsuperscript{25–27} These effects were mediated by the AT\(_1\) receptor and were induced by an activation of membrane-bound NAD(P)H oxidase because the flavoprotein inhibitor diphenylene iodium and p22phox antisense oligonucleotides attenuated this response.\textsuperscript{27–30} AT\(_1\) receptor–transduced ROS formation, depending on NAD(P)H oxidase, has also been observed in several renal cells in culture.\textsuperscript{27} Similar observations have been made in the kidney when the endogenous renin-angiotensin system was stimulated using the two kidney–one clip (2K-1C) model.\textsuperscript{31} Pharmacologic inhibitor studies of vascular homogenates from 2K-1C animals demonstrated that the major source of \( \cdot \mathrm{O}_2^- \) was a NAD(P)H oxidase that was activated by a protein kinase C–dependent mechanism.\textsuperscript{31} Inhibition of ROS in models of AngII infusion or 2K-1C partly attenuates hypertension, indicating that AngII-induced ROS is important for vasoconstriction in these models.\textsuperscript{10}

The mechanisms by which AngII activates NAD(P)H oxidase has been the subject of active research. In vascular cells, caveolin 1 (a component of caveolae/lipid rafts that are cholesterol-enriched specialized membrane microdomains) is necessary for AngII-mediated Rac1 and NAD(P)H oxidase activation and ROS generation.\textsuperscript{32} Principally, it has been found that AngII stimulates upregulation of various NAD(P)H oxidase subunits, including Nox1, p47phox, p67phox, and p22phox, in various cell types.\textsuperscript{33–38} Functional evidence that this induction of enzyme subunits is important comes from experiments interfering with the AngII-induced expression of subunits by antisense or small-inhibitory RNA technology.\textsuperscript{27,28,34} In addition, some limited studies have confirmed these findings in knockout mice. For example, AngII infusion leads to blunted ROS formation and an attenuated BP response in reduced Nox1-deficient mice.\textsuperscript{39} Similarly, mice deficient in the p47phox gene showed a reduced ROS formation and lower arterial BP during AngII infusion.\textsuperscript{36} In contrast to AT1 receptor activation, it seems that AngII-mediated stimulation of AT2 receptors downregulates several NAD(P)H oxidase components (Nox1, p22phox, and p67phox).\textsuperscript{3} Conversely, AngII facilitates assembly of NAD(P)H oxidase subunits (Figure 2). AngII has been found to induce serine phosphorylation of p47phox, resulting in an increased binding of p47phox to p22phox.\textsuperscript{40} AngII also stimulates Rac1 by disrupting the binding of Rac to the GDP dissociation inhibitor RhoGDI. Rac1 in turn binds to and activates Nox4, increasing \( \cdot \mathrm{O}_2^- \) generation.\textsuperscript{41,42}

Aldosterone can also induce ROS formation by increasing expression of the p47phox and p67phox subunits of NAD(P)H oxidase.\textsuperscript{43,44} In fact, some of the effects previously attributed to AngII on NAD(P)H oxidase–mediated \( \cdot \mathrm{O}_2^- \) formation may be due to the secondary release of aldosterone. ROS formation in the heart induced by AngII infusion was prevented by the mineralocorticoid receptor antagonist spironolactone and depended on the presence of Nox2.\textsuperscript{45}

**DOES ANGII STIMULATE ROS FORMATION BY SYSTEMS OTHER THAN NAD(P)H OXIDASE?**

Recent evidence suggests that AngII stimulates mitochondrial ROS generation through the opening of mitochondrial K\(_{\text{ATP}}\) channels, leading to redox-sensitive activation of mitogen-activated protein kinases (MAPK).\textsuperscript{46} It is interesting that a process of AngII-mediated preconditioning has been described, at least in cardiac myocytes, in which NAD(P)H oxidase–derived \( \cdot \mathrm{O}_2^- \) stimulated K\(_{\text{ATP}}\) channels, facilitating the efflux of large mitochondrial-derived amounts of \( \cdot \mathrm{O}_2^- \) into the cytoplasm.\textsuperscript{47,48} The relationship between generation of \( \cdot \mathrm{O}_2^- \) in the mitochondrial respiratory chain and NAD(P)H oxidase is complex, and it has been shown that mitochondrial inhibitors suppress the induction of Nox1.\textsuperscript{49}

A more indirect effect of AngII on ROS formation may be mediated through the hypoxia-inducible factor 1-α (HIF-1α). We previously showed that AngII stimulates HIF-1α expression through AT2 receptors via suppression of prolyl hydroxylase 3, an enzyme that hydroxylates HIF-1α with the consequence of inducing degradation of this factor.\textsuperscript{50,51} In heterozygous mice partially
deficient in HIF-1α, ROS formation induced by chronic intermittent hypoxia was, in contrast to wild-type mice, absent, indicating a role of HIF-1α in the formation of ROS.52 However, a vice versa pathway has also been described, because ROS interact with and inhibit prolly hydroxylase, leading to a decrease in HIF-1α hydroxylation and stabilization of this transcription factor.53

**FUNCTIONAL ROLE OF ANGII-INDUCED ROS**

ROS are involved in many pathways, and potential molecular targets are summarized in Figure 3. MAPK are activated by ROS. AngII-induced p38 MAPK activation in vascular smooth muscle cells depends on H2O2,54 We found that AngII stimulates phosphorylation and activation of extracellular signal–regulated kinase 1,2 (ERK1,2) in renal tubular cells depending on NAD(P)H oxidase–mediated -O2− formation.55 AngII-mediated phosphorylation of the EGF receptor is sensitive to inhibition by antioxidants, presumably by interfering with the proteolytic cleavage of heparin-binding EGF that transactivates the EGF receptor.56 Src tyrosine kinases are also activated by AngII-stimulated ROS formation. Various tyrosine phosphatases are susceptible to oxidation and inactivation by ROS because these enzymes contain a conserved cysteine that is oxidized to sulfenic acid in the presence of ROS.57 In addition, transcription factors such as NF-κB and AP-1 are induced by oxidative stress.58

Matrix metalloproteinases 2 and 9, key enzymes in the degradation of extracellular matrix components, are activated by ROS.59 Substantial experimental evidence indicates that AngII-induced ROS are potential regulators of adhesion molecule expression. Induction of intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1 by AngII involves NAD(P)H oxidase activation.60 Together with NF-κB–mediated transcriptional activation of chemokines, this induction of adhesion molecules contributes to renal inflammation. ROS also activate ion channels (Ca2+ and K+) and modulate vascular tone through this mechanism.61

AngII-mediated ROS formation is important for renal growth processes that are part of an adaptive process of surviving nephrons during chronic renal injury.62 We intensively studied AngII-dependent growth of renal cells. AngII induces hypertrophy of cultured mouse proximal tubular cells, and this hypertrophy is associated with arrest of the G1 phase.63–64 This AngII-mediated cell-cycle arrest depends on induction of p27Kip1, an inhibitor of G1 phase cyclin-dependent kinase (CDK)/cyclin complexes.65–67 AngII stimulates the accumulation of -O2− in tubular cells by upregulation of p22phox.66 The ROS, in turn, activate ERK1,2, and MAPK.55 Accordingly, we consequently asked whether the p44/42 MAPK may in turn directly phosphorylate p27Kip1. Activated ERK1,2 directly phosphorylates the recombinant p27Kip1 in vitro.64 Functional studies showed that serine178 mutation, in contrast to wild-type or serine,10 and threonine187 mutations of p27Kip1 failed to promote hypertrophy, showing the role for specific protein phosphorylation in mediating the effect of AngII on cell hypertrophy.68 Although primarily derived from cell culture studies, similar mechanisms are operative in vivo.69 Infusion of AngII into naive rats (rate of 250 ng/min into male Wistar rats [body weight 200 g]) for 7 d increases formation of -O2− in tubular cells and stimulates protein expression of p27Kip1.69 The infusion of AngII concomitantly reduces tubular proliferation, indicating G1 phase arrest. Immunoprecipitation experiments revealed that the increased p27Kip1 protein associates with G1- phase arrest.69 Co-administration of the radical scavenger dimethylthiourea eliminated this AngII-mediated p27Kip1 expression without reducing systemic BP.69 Other work, mainly performed on endothelial and smooth muscle cells, points to a role of AngII-induced ROS in cell migration and apoptosis.70 Table 1 shows various experimental models of renal disease in which AngII-mediated ROS formation has been implicated, mainly by administration of antioxidants or inhibition of NAD(P)H oxidase activation.

**WHAT IS THE ROLE OF ANGII-MEDIATED ROS FORMATION IN HUMAN RENAL DISEASE?**

Although numerous experimental studies have indicated a role of AngII-mediated ROS formation, extending this concept to the pathophysiology and treatment of human renal disease has been problematic. Several descriptive studies on oxidative stress in various renal diseases exist. Women with pre-eclampsia have an agonistic antibody in the plasma that binds to AT1 receptors that activate NAD(P)H oxidase by inducing p22phox, p47phox, and p67phox.71 We and others have found in biopsies from patients with different types of renal allograft rejection signs of oxidative stress associated with the apoptosis of renal cells.72,73 In general, human studies using vitamin E as an antioxidant have failed to show any benefit, and some data from cardiovascular studies even suggest that this treatment may be harmful.74–76 Part of this problem may be that when vitamin E or other antioxidants scavenge -O2−, they become
radicals themselves that have their own effects. N-acetylcysteine, a potential antioxidant, has been widely used in studies to prevent contrast media–induced renal injury. However, the current data are controversial, and it is unclear whether the effects are really due to scavenging of ROS or rather represent direct inhibition of NAD(P)H oxidase or by facilitating preassembly of the NADPH oxidase complex. Recent studies also suggest that mitochondrial ROS generation is stimulated by AngII. -O2− is involved in several signal pathways, and redox-sensitive transcriptional factors, including AP-1 and NF-κB, suggest that ROS are an important second messenger of AngII’s transcriptional effects. The concept of oxidative stress has changed insofar as early discrete changes occur within the cell or even an enzyme system without a necessary modification of total cellular redox status. These subtle changes may already influence the genetic program of the renal cells, leading to an altered transcriptome. AngII-induced ROS may play a pivotal role in several pathophysiologic diseases of the kidney and vasculature (e.g., glomerulonephritis, diabetic nephropathy, hypertension, acute renal failure, progression of renal disease). Although inhibition of the RAAs in humans leads to a reduction in markers of oxidative stress, antioxidative interventions targeting on the level of total cellular redox status (e.g., antioxidative vitamins) have been disappointing. Presumably, therapeutic approaches might better focus on repairing or inhibiting the function of individual enzymes [e.g., NAD(P)H oxidase] involved in ROS generation. Interestingly side effects of drugs such as mycophenolate acid that have been shown to inhibit NAD(P)H oxidase may serve as paradigm for further development of antioxidative strategies to combat kidney diseases.

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CONCLUSIONS

Experimental studies provide ample evidence that AngII stimulates intracellular formation of -O2− by upregulating subunits of the membrane-bound NAD(P)H oxidase and by facilitating assembly of subunits. Recent studies also suggest that mitochondrial ROS generation is stimulated by AngII. -O2− is involved in several signal pathways, and redox-sensitive transcriptional factors, including AP-1 and NF-κB, suggest that ROS are an important second messenger of AngII’s transcriptional effects. The concept of oxidative stress has changed insofar as early discrete changes occur within the cell or even an enzyme system without a necessary modification of total cellular redox status. These subtle changes may already influence the genetic program of the renal cells, leading to an altered transcriptome. AngII-induced ROS may play a pivotal role in several pathophysiologic diseases of the kidney and vasculature (e.g., glomerulonephritis, diabetic nephropathy, hypertension, acute renal failure, progression of renal disease). Although inhibition of the RAAs in humans leads to a reduction in markers of oxidative stress, antioxidative interventions targeting on the level of total cellular redox status (e.g., antioxidative vitamins) have been disappointing. Presumably, therapeutic approaches might better focus on repairing or inhibiting the function of individual enzymes [e.g., NAD(P)H oxidase] involved in ROS generation. Interestingly side effects of drugs such as mycophenolate acid that have been shown to inhibit NAD(P)H oxidase may serve as paradigm for further development of antioxidative strategies to combat kidney diseases.

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Table 1. Evidence for a potential role of ROS in experimental renal disease

| Ischemia-reperfusion models of acute renal failure |
| Anti-Thy1 glomerulonephritis |
| Anti–glomerular basement membrane antibody models |
| Passive Heymann nephritis |
| Puromycin aminonucleoside–induced minimal-change disease |
| Diabetic nephropathy |
| Hypertension induced by AngII infusion |
| Hypertension induced by chronic NO inhibition |
| 2-kidneys–1-clip hypertension |
| Hyperlipidemia–induced renal damage |
| Renal ablation models |
| Calcineurin inhibitor toxicity |
| Acute and chronic transplant nephropathy |


