
Angiotensin II and Oxidative Stress

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Abstract There is an increasing body of evidence that oxidative stress markedly contributes to endothelial dysfunction and to poor prognosis in patients with coronary artery disease and hypertension. Among many stimuli for oxidative stress in vascular tissue, the role of angiotensin II has been extensively studied. When given acutely, angiotensin II stimulates the release of nitric oxide and activates a nonphagocytic NAD(P)H oxidase, leading to formation of superoxide and of the nitric oxide/superoxide reaction product peroxynitrite. Peroxynitrite in turn may cause tyrosine nitration of prostacyclin synthase and/or oxidation of zinc–thiolate complexes of the nitric oxide synthase associated with inhibi-

tion or uncoupling of these enzymes. Further, angiotensin II causes endothelial dysfunction, hypertension, increases the endothelin expression, and inhibition of the cGMP-cGK-I signaling cascade, all of which are secondary to angiotensin II-stimulated production of reactive oxygen species.

Keywords Angiotensin II · NADPH oxidase · Endothelium · Peroxynitrite · Superoxide

Abbreviations

ACE	Angiotensin converting enzyme
ADMA	Asymmetric dimethylarginine
AT-1	Angiotensin type 1 receptor
BH ₄	Tetrahydrobiopterin
cGK	cGMP-dependent protein kinase
cGMP	Cyclic guanosine monophosphate
DDAH	Dimethylarginine dimethyl-aminohydrolase
H ₂ O ₂	Hydrogen peroxide
LDL	Low-density lipoprotein
L-NAME	N ^G -nitro-L-arginine methyl ester
L-NNA	N ^G -nitro-L-arginine
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NOS III	Nitric oxide synthase
nox	Nonphagocytic NAD(P)H oxidase
O ₂ ^{•-}	Superoxide
ONOO ⁻	Peroxynitrite
OH	Hydroxyl radical
PDE 1A1	Phosphodiesterase type 1A1
PKC	Protein kinase C
PRMT	Protein arginine methyltransferase
P-VASP	Phosphorylated vasodilatory stimulated phosphoprotein
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase

1

Introduction

In the last decade, a great deal has been learned about the critical role of reactive oxygen species (ROS) in the pathophysiology of vascular disease. The effects of angiotensin II on ROS production and its functional consequences for endothelial function, hypertension, endothelin expression and activation of superoxide (O₂^{•-}) producing enzymes such as the NAD(P)H oxidase, have been studied

extensively. In this review, mechanisms by which angiotensin II-mediated ROS production affects vascular function will be discussed.

2

Endothelial Dysfunction, Oxidative Stress and Prognosis

Traditionally, the role of the endothelium was thought to be primarily that of a selective barrier to the diffusion of macromolecules from the blood lumen into interstitial space. During the past 20 years, numerous additional roles for the endothelium have been defined such as regulation of vascular tone, modulation of inflammation, promotion as well as inhibition of vascular growth, and modulation of platelet aggregation and coagulation. Endothelial dysfunction is characteristic of patients with apparent coronary atherosclerosis or patients with cardiovascular risk factors such as hypercholesterolemia, hypertension, diabetes and chronic smoking. Recent studies indicate that assessment of endothelial function in both the coronary and peripheral circulation provide important prognostic information concerning future cardiovascular events (Al Suwaidi et al. 2001; Heitzer et al. 2001). Examination of patients with mild coronary artery disease and endothelial dysfunction, at the level of coronary conductance and vessel resistance, demonstrated a greater incidence of cardiovascular events compared to patients with better endothelial function (Schachinger et al. 2000). More recent studies indicate that endothelial dysfunction in patients with angiographically normal coronary arteries was predictive of subsequent cardiovascular events (Halcox et al. 2002). Although the mechanisms underlying endothelial dysfunction may be multifactorial, there is a growing body of evidence that increased production of free radicals may considerably contribute to this phenomenon. In patients with coronary artery disease, the extent of vitamin C-induced improvement of endothelial dysfunction was a strong and independent predictor of subsequent cardiovascular events (Heitzer et al. 2001). This indicates that high oxidative stress in vascular tissue not only contributes to endothelial dysfunction, but also decisively determines the prognosis in patients with cardiovascular risk factors.

3

Oxidative Stress

3.1

Reactive Oxygen Species

ROS are molecules that are initially derived from oxygen but have undergone univalent reduction, so that they readily react with other biological products. ROSs include $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2), hydroxyl radical (OH), nitric oxide (NO) and the $NO/O_2^{\cdot-}$ reaction product peroxynitrite. Of these, $O_2^{\cdot-}$, H_2O_2 , NO and $ONOO^-$ are formed in response to angiotensin II treatment and modify the activity of signaling proteins and enzymes. In contrast to $O_2^{\cdot-}$, NO, OH,

H_2O_2 , ONOO^- and hypochlorous acid are not free radicals per se, but have oxidizing effects that markedly contribute to oxidative stress. Superoxide is dismutated by superoxide dismutase (SOD) to the more stable radical H_2O_2 . Catalase and glutathionperoxidase are important scavengers of H_2O_2 , leading to the formation of water. The rapid bimolecular reaction between NO and $\text{O}_2^{\cdot-}$ to yield ONOO^- is more than three times faster than the enzymatic dismutation of $\text{O}_2^{\cdot-}$ catalyzed by SOD. Thus, ONOO^- formation represents a major potential pathway for NO reactivity, which depends on rates of tissue $\text{O}_2^{\cdot-}$ production. ONOO^- has a cytotoxic potential about 1,000 times higher than that of H_2O_2 , and has a low stimulatory capacity of the target enzyme guanylyl cyclase. ONOO^- will also protonate to peroxynitrous acid (ONOOH), to yield an oxidant with the reactivity of $\cdot\text{OH}$, via metal independent mechanisms. Peroxynitrite in pure form will cause oxidative damage to protein, lipid, carbohydrate, DNA, subcellular organelles, and cell systems. Peroxynitrite may also cause tyrosine nitration of prostacyclin synthase (Zou et al. 1999) and oxidation of the zinc-thiolate complex of NOS (Zou et al. 2002) leading to uncoupling or inhibition of targeted enzymes.

3.2

Angiotensin and ROS Production

3.2.1

Endothelium

Incubation of cultured rat aortic endothelial cells with angiotensin II stimulates release of NO (Pueyo et al. 1998). These actions are mediated by the AT_1 receptor as confirmed by their inhibition with the AT_1 receptor antagonist losartan. For example, angiotensin II-stimulated cGMP production by reporter cells was prevented by a specific calmodulin antagonist, suggesting that angiotensin II stimulated endothelial calmodulin-dependent NOS (Pueyo et al. 1998). In porcine pulmonary endothelial cells, there is evidence that angiotensin IV rather than angiotensin II contributes to NO release, since an angiotensin IV antagonist blocked both angiotensin II- and angiotensin IV-induced NO production (Hill-Kapturczak et al. 1999). Angiotensin II also stimulates the production of $\text{O}_2^{\cdot-}$, which is mediated by the AT_1 receptor (Sohn et al. 2000). There is also evidence that stimulation of the AT_2 subtype functionally antagonizes AT_1 receptor-induced $\text{O}_2^{\cdot-}$ production, involving a tyrosine phosphatase pathway (Sohn et al. 2000). The simultaneous release of NO and $\text{O}_2^{\cdot-}$, in response to angiotensin II stimulation, has been recently demonstrated in vitro (Pueyo et al. 1998) as well as in humans (Dijkhorst-Oei et al. 1999). In cultured endothelial cells, angiotensin II stimulated the formation of ONOO^- , as indicated by the increase in luminol-dependent chemiluminescence and by the inhibitory effects of SOD and NOS III inhibitors on chemiluminescence signals (Pueyo et al. 1998). By using venous occlusion plethysmography, Tonk Rabelinks' group showed that in a NO-free system (achieved by N^G -monomethyl-L-arginine treatment), angioten-

sin II-induced vasoconstriction was significantly enhanced in the forearm circulation of healthy volunteers. Likewise, angiotensin II-induced vasoconstriction was greatly diminished in response to concomitant treatment with vitamin C, suggesting that angiotensin II simultaneously stimulates NO and $O_2^{\cdot-}$ production in humans (Dijkhorst-Oei et al. 1999).

3.2.2

Vascular Smooth Muscle

Incubation of cultured smooth muscle cells with angiotensin II leads to a marked increase in the production of $O_2^{\cdot-}$. The likely $O_2^{\cdot-}$ source was identified as a NADH/NADPH driven oxidase (Griendling et al. 1994). This assumption was based on inhibitor experiments where diphenylene iodonium (inhibitor of flavin-containing oxidases, DPI) and quinacrine inhibited NADH and NADPH-stimulated $O_2^{\cdot-}$ production (Griendling et al. 1994). Further studies demonstrated that $O_2^{\cdot-}$ production induced by angiotensin II was inhibited by *N*-acetylcysteine and by the free radical scavenger tiron (Laursen et al. 1997). Angiotensin II-induced formation of the $O_2^{\cdot-}$ -dismutation product H_2O_2 can be detected within minutes (Ushio-Fukai et al. 1996). Both antisense against the p22^{phox} NADPH oxidase subunit and overexpression of catalase prevent angiotensin II-stimulated formation of H_2O_2 (Ushio-Fukai et al. 1996). Intracellular signaling mechanisms by which angiotensin II stimulates ROS formation is still under debate, and may involve arachidonic acid made by phospholipase A₂, or indirectly via phospholipase D-mediated degradation of phosphatidylcholine to phosphatidic acid (Griendling et al. 1994).

Most of the experiments to detect $O_2^{\cdot-}$ production in homogenates use the chemiluminescence substance lucigenin. Lucigenin has been demonstrated to undergo redox cycling when used in high concentrations (250 μ M), and in particular when NADH (Janiszewski et al. 2002) is used as a substrate. These limitations do not occur when low concentrations of lucigenin (5 μ M) (Li et al. 1998) and NADPH as substrate are used (Griendling et al. 2000). Recent studies using electron paramagnetic resonance to detect $O_2^{\cdot-}$ produced by cultured smooth muscle cells, in response to angiotensin II stimulation, revealed that NADPH is the major substrate for oxidase (Sorescu et al. 2001). In cultured endothelial cells, both NADPH and NADH were equally effective in stimulating superoxide production (Somers et al. 2000). Thus, the question whether NADH and/or NADPH are the preferred substrate for this enzyme is still under debate.

4 Effects of Angiotensin II

4.1 Activation of Oxidases

4.1.1 Effects of Angiotensin II on the Activity and Expression of the Nonphagocytic NAD(P)H Oxidase

Although vascular NAD(P)H oxidase is quite similar to the phagocytic multi-component NAD(P)H oxidase, there are several distinct features that allow differentiation between both oxidases (Griendling et al. 2000). Activation occurs within seconds with the phagocytic oxidase, and within minutes or hours with the nonphagocytic oxidase (see also Table 1). The $O_2^{\cdot-}$ -producing capacity of the phagocytic oxidase is in the micromolar range, and $O_2^{\cdot-}$ is being released in a burst-like fashion. In contrast, a steady release of low amounts of $O_2^{\cdot-}$, in the nanomolar range, is produced by vascular NADPH oxidase. Endothelial and adventitial cells, as well as inflammatory cells such as neutrophils and macrophages, express NAD(P)H oxidase consisting of the flavocytochrome b_{558} subunits gp91^{phox} and p22^{phox}, the cytosolic factors p47^{phox} and p67^{phox}, and the small GTPase rac1 (Griendling et al. 2000). In contrast to endothelial, adventitial and inflammatory cells, smooth muscle cells lack gp91^{phox}; however, recent studies identified the existence of two gp91^{phox} homologs, namely nox1 and nox4 (Lambeth et al. 2000; Suh et al. 1999). Because gp91^{phox} and nox harbor electron transfer moieties of the enzyme and thus serve as the catalytic component, regulation of these subunits is of utmost importance to the ultimate functioning of the enzyme.

In vitro, incubation of endothelial and smooth muscle cells with angiotensin II resulted in increases in activity and expression of NADPH oxidase subunits

Table 1 Characteristics of phagocytic and vascular NAD(P)H oxidases

	Phagocytic oxidase	Vascular oxidase
Substrate K_m	NADPH (30 μM)	NADPH (50 μM) NADH (10 μM)
Kinetics of activation	Seconds	Minutes to hours
Capacity	130 nmol $O_2^{\cdot-}/min \cdot mg$ cell protein	40 nmol $O_2^{\cdot-}/min \cdot mg$ cell protein
Fate of released $O_2^{\cdot-}$	Extracellular	Intracellular and/or extracellular
Cellular location	Phagosomes	Plasma membrane/microsomes
Subunit structure	gp91 ^{phox} p22 ^{phox} p47 ^{phox} p67 ^{phox} rac-1/rac-2	Endothelial cells and fibroblasts: gp91 ^{phox} , p22 ^{phox} , p47 ^{phox} p67 ^{phox} , rac-1/ rac-2 Vascular smooth muscle cells: nox1, nox4, p22 ^{phox} , p47 ^{phox} , p67 ^{phox} , rac-1/ rac-2

such as gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, nox1 and nox4 (Touyz et al. 2002; Wingler et al. 2001). Antisense directed against p22^{phox} and nox-1 resulted in an inhibition of angiotensin II-induced ROS formation (Lassegue et al. 2001), while antisense against nox4 resulted in a decrease in basal O₂⁻ production (Szocs et al. 2002). In vivo treatment with angiotensin II increased expressions of p22^{phox} (three- to fourfold), gp91^{phox} (threefold), p47 and p67^{phox} in endothelial and smooth muscle cells, and the adventitia, respectively (Cifuentes et al. 2000; Di Wang et al. 1999; Mollnau et al. 2002; Pagano et al. 1998; Pagano et al. 1997). Likewise, in experimental hypercholesterolemia, increased activity of the NAD(P)H oxidase was linked with increased AT₁ receptor expression, and the activity of the enzyme was reduced by in vivo AT₁ receptor blockade (Warnholtz et al. 1999). Evidence for a role of the RAAS in the regulation of gp91^{phox} isoform expression in smooth muscle cells was provided by Mollnau et al. (2002) and Wingler et al. (2001), who demonstrated increased expression of nox1 and nox4 in aortas from hypertensive, angiotensin II-infused animals and in transgenic rats, overexpressing the Ren2 gene.

Further insight into the role of NAD(P)H oxidase in vascular disease was provided by knockout experiments. Infusion of angiotensin II into wild-type mice increased O₂⁻ production, blood pressure, media viscosity, gp91^{phox} expression and nitrotyrosine content (footprint for ONOO⁻-induced vascular damage) in vascular tissue, all of which were significantly reduced in gp91^{phox} knockout animals (Wang et al. 2001). Infusion of angiotensin II caused significantly greater increases in vascular O₂⁻ production in aortas from wild-type mice as compared to aortas from p47^{phox} knockout animals (Brandes et al. 2002). Likewise, infusion of angiotensin II at subpressor doses caused myocardial hypertrophy in wild-type, but not in gp91^{phox} knockout mice, suggesting an involvement of myocardial NAD(P)H oxidase in mediating myocardial hypertrophy independent of changes in blood pressure (Bendall et al. 2002). These data clearly indicate a crucial role for the NAD(P)H oxidase in mediating angiotensin II-induced increases in oxidative stress, in vascular and myocardial tissue.

4.1.2

Angiotensin II and the Nitric Oxide Synthase

As mentioned above, angiotensin II stimulates the simultaneous release of NO and O₂⁻ leading to ONOO⁻ formation (Pueyo et al. 1998). Long-term incubation of pulmonary endothelial cells with angiotensin II markedly increased NOS III expression at the mRNA and protein levels, with a maximum after 8 h (+300%) (Olson et al. 1997). These effects were mediated by the AT-1 subtype, since pretreatment with losartan abolished the effects on NOS III expression. Despite the marked increases in NOS III expression, angiotensin II treatment usually led to significant endothelial dysfunction (Mollnau et al. 2002). The discrepancy between the increases in NOS III expression and vascular O₂⁻ production may indicate that NOS III is itself a significant O₂⁻ source, under these conditions. Recent results from in vivo studies in angiotensin II-treated, hypertensive animals

confirm this concept. At both the RNA and protein levels, NOS III enzyme was up-regulated more than twofold in the myocardium (Tambascia et al. 2001) and vessels (Mollnau et al. 2002; Sullivan et al. 2002) of angiotensin II-treated animals as compared to controls. Nevertheless, there was considerable endothelial dysfunction as well as a marked decrease in vascular NO bioavailability in angiotensin II-treated animals, indicating that vascular $O_2^{\cdot-}$ from hypertensive animals may either overwhelm nitric oxide production of up-regulated NOS III, or that the up-regulated NOS III itself is uncoupled, thereby contributing to $O_2^{\cdot-}$ production.

Conditions leading to NOS III uncoupling, such as BH_4 deficiency (NOS III cofactor) (Xia et al. 1998) and intracellular L-arginine (NOS III substrate) (Gorren et al. 1998) depletion, have been characterized. In BH_4 deficiency, electrons flowing from the NOS III reductase domain to the oxygenase domain are diverted to molecular oxygen, rather than to L-arginine, resulting in production of $O_2^{\cdot-}$ rather than NO. What conditions are responsible for NOS III uncoupling in vessels from angiotensin II-infused animals? In vitro angiotensin II treatment increased the vascular formation of the NO/ $O_2^{\cdot-}$ reaction product, ONOO⁻ (Pueyo et al. 1998). Peroxynitrite in turn has been shown to rapidly oxidize the active NOS III cofactor BH_4 to inactive molecules, e.g., BH_2 switching NOS III from a NO to an $O_2^{\cdot-}$ -producing enzyme (Laursen et al. 2001). An alternative explanation whereby ONOO⁻ may cause NOS III uncoupling was recently reported by Zou et al (2002). Using cultured endothelial cells, ONOO⁻ was shown to directly cause oxidation of the zinc-thiolate complex within the enzyme, all of which may favor NOS III monomer over dimer formation, leading to NOS III uncoupling. Likewise, Jennifer Pollock's group demonstrated increased NOS III expression in vessels from angiotensin II-infused animals (Sullivan et al. 2002). Interestingly, a considerable portion of the increased NOS III protein was located in the cytosolic fraction. Since NOS III in its activated state is a membrane-associated enzyme (targeted to caveolae and golgi membranes via N-myristoylation and palmitoylation processes), redistribution toward the cytosol and therefore the altered subcellular location of NOS III may also contribute to endothelial dysfunction and NOS III uncoupling in this particular animal model.

As pointed out above, another mechanism leading to NOS III uncoupling is depletion of intracellular L-arginine. Recent in vitro and in vivo studies demonstrate that in almost all situations where oxidative stress is encountered in vascular tissue, intracellular and plasma concentrations of asymmetric dimethylarginines (ADMA) are increased (Boger et al. 2000a; Boger et al. 1998). This phenomenon may be explained either by an increase in the activity of methylating enzymes such as S-adenosylmethionine-dependent methyltransferases (PRMT Type I) (Boger et al. 2000b), or a decrease in the activity of ADMA demethylating enzymes such as dimethylarginine dimethylaminohydrolase (DDAH); all of which result in increased intracellular production of ADMA. In concentrations reached by stimulation of methyltransferases or inhibition of DDAH, ADMA has been shown to significantly inhibit NOS III activity. Interestingly, the activity of both enzymes regulating intracellular ADMA concentra-

tions has been reported to be redox-sensitive. Oxidative stress increases the activity of methylating enzyme such as PRMT I (Boger et al. 2000b), while decreasing the activity of demethylating DDAH (Ito et al. 1999). These observations may explain why in angiotensin II hypertension, L-arginine is able to improve endothelial dysfunction in experimental animals (Pucci et al. 1995), even when intracellular L-arginine levels are not decreased (the so-called L-arginine paradox). Recent studies with patients diagnosed with essential hypertension revealed increased plasma levels of ADMA (Surdacki et al. 1999). Interestingly, treatment with ACE-inhibitors, AT₁ receptor blockers, but not beta-blockers, reduced plasma ADMA levels. However, all drugs were able to normalize blood pressure, pointing to a role for the RAAS in mediating increased plasma ADMA levels in hypertensive patients (Ito et al. 2001).

How can we assess NOS III uncoupling in vascular tissue? Pritchard et al. previously demonstrated that NOS III inhibition in endothelial cells cultured with L-NNA increased steady state O₂⁻ levels (Vasquez-Vivar et al. 1998). These findings indicate that a large portion of baseline O₂⁻ production is scavenged due to its interaction with NO. After incubation of endothelial cells with native LDL, O₂⁻ levels increased markedly, a phenomenon which largely was blocked by L-NAME (Pritchard et al. 1995). Reduction of steady state O₂⁻ levels by means of NOS III inhibition identified NOS III as an important O₂⁻ source. The assumption that NOS III is uncoupled in angiotensin II hypertension was strengthened recently by experiments with the NOS inhibitor L-NNA. Vascular O₂⁻ production was assessed using lucigenin- and coelenterazine-derived chemiluminescence. In control vessels with an intact endothelium, NOS-inhibition with L-NNA increased vascular O₂⁻, indicating that basal production of endothelium-derived nitric oxide quenches the baseline chemiluminescence signal. In contrast, incubation of aortas, from angiotensin II-treated animals, with L-NNA markedly reduced the chemiluminescence signal, thus indicating that NOS III is an important O₂⁻ source (Mollnau et al. 2002). It seems that in most situations where high oxidative stress is encountered, NOS III is in an uncoupled state as observed in the experimental animal (Hink et al. 2001; Laursen et al. 2001; Oelze et al. 2000) and in patients with hypercholesterolemia (Stroes et al. 1997) and diabetes (Heitzer et al. 2000b) and in chronic smokers (Heitzer et al. 2000a).

4.1.3 Angiotensin II and the Xanthine Oxidase

Xanthine oxidase is an oxidoreductase that catalyzes the oxidation of hypoxanthine and xanthine in purine metabolism. This enzyme exists in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase. Recent studies with double-transgenic rats (dTGR) harboring human renin and angiotensinogen genes provide evidence for angiotensin II involvement in the regulation of xanthine oxidase-mediated O₂⁻ production (Mervaala et al. 2001). Incubation of vascular tissue with SOD and the xanthine oxidase inhibitor

oxypurinol, improved endothelial dysfunction. Increased production of 8-iso-prostaglandin F₂ α , decreased NO production, increased xanthine oxidase activity in the kidney, and endothelial dysfunction were corrected by in vivo treatment with the angiotensin II (type 1) receptor blocker valsartan, pointing to a role for angiotensin II xanthine oxidase activation in this particular animal model.

4.2

Effects of Angiotensin II-Induced Hypertension on cGMP, sGC and cGK-I Signaling

Vessels from animals with angiotensin II-evoked hypertension show not only reduced vasodilation to endothelium-dependent vasodilators, but also to endothelium-independent vasodilators such as SNP and NTG (Rajagopalan et al. 1996). Reduced nitrovasodilator responses, in vessels from angiotensin-infused hypertensive animals, may be explained by the reduced expression of both sGC subunits (Mollnau et al. 2002). This observation complements reports from other animal models of hypertension, where the expression of one or both sGC subunits (α_1 and β_1) and/or NO-dependent sGC activity were significantly decreased (Jacke et al. 2000; Kloss et al. 2000; Lopez-Farre et al. 2002; Ruetten et al. 1999). In all these animal models, endothelial dysfunction was associated with enhanced vascular ROS formation. Normalization of blood pressure by hydralazine-treatment (Bauersachs et al. 1998), vitamin C treatment (Marques et al. 2001), in vivo PKC-inhibition, Ca²⁺-antagonist or chronic ACE-inhibitor treatment (Jacke et al. 2000), normalized or even enhanced sGC expression. The question remains whether the high blood pressure or the observed increases in ROS production within smooth muscle cells represents the crucial stimulus for a decrease in sGC expression. Georg Kojda's group recently showed that incubation of vascular tissue with the sGC inhibitor LY83583, a redox-cycler that generates intracellular O₂⁻, mimicked the changes observed in vessels from cholesterol-fed animals: that is, sGC β_1 subunit expression was up-regulated. However, in contrast to hypercholesterolemic animals, NO-dependent activity of sGC, in aortic cytosol, was abolished (Laber et al. 2002). Thus, it appears that the observed decrease in sGC expression, in vessels from angiotensin II-treated animals, was mainly elicited by high blood pressure and not linked to increased O₂⁻ formation. Infusion of angiotensin II did not modify cGK I expression. However, cGK I activity, as assessed by phosphorylation of the cGK-I substrate, the vasodilatory stimulated phosphoprotein (P-VASP), was markedly reduced, suggesting a signaling defect upstream of cGK I (Mollnau et al. 2002). This was supported by the fact that in vivo treatment with chelerythrine reduced oxidative stress and improved both endothelial dysfunction and P-VASP (M. Oelze, unpublished observation). Angiotensin II increased the activity and expression of the PDE1A1 isoform in cultured smooth muscle cells, providing evidence that increased cGMP metabolism may partly contribute to endothelial dysfunction

and decreased cGK I activity, as observed in angiotensin II-induced hypertension (Kim et al. 2001).

4.3

Key Role for Protein Kinase C in Angiotensin II Induced $O_2^{\cdot-}$ Production

Recent in vitro studies revealed that angiotensin II down-regulates nox4, while markedly up-regulating the nox1 isoform (Lassegue et al. 2001). Up-regulation of nox1 was also observed in response to the phorbol ester, phorbol myristate acetate (a direct activator of PKC). Angiotensin II-induced up-regulation of nox1 was inhibited by specific inhibitors of PKC, suggesting a crucial role for PKC in the up-regulation of the nox1 message (Gorlach et al. 2000). Antisense nox1 mRNA completely inhibited angiotensin II-induced $O_2^{\cdot-}$ production, supporting a role for nox1 in redox signaling in vascular smooth muscle cells (Lassegue et al. 2001). The results obtained from our recent in vivo experiments support this concept. Using a nonradioactive PKC assay, we found a twofold increase in whole vessel homogenates from angiotensin II-treated animals, as compared with controls. While nox4 expression was only slightly modified by angiotensin II treatment, dramatic increases in the expressions of nox1 (~sevenfold), gp91^{phox} (threefold) and p22^{phox} (threefold) were observed. In vitro and in vivo treatment with the PKC inhibitor chelerythrine markedly reduced angiotensin II-stimulated $O_2^{\cdot-}$ production and prevented up-regulation of nox1 (Mollnau et al. 2002). An interesting aspect of this particular study was that the in vivo PKC inhibition also prevented the up-regulation of p22^{phox}, suggesting that PKC may function as a general regulator of oxidase function by regulating both activity and expression. Interestingly, recent in vitro studies indicate that $O_2^{\cdot-}$ is a strong stimulus for PKC activation (Nishikawa et al. 2000). Thus, PKC increases oxidative stress in angiotensin II-treated vascular tissue by stimulating NADPH-oxidase mediated $O_2^{\cdot-}$ production, and by its activation in response to oxidative stress in a positive feedback fashion.

4.4

Angiotensin II, Oxidative Stress, Endothelin Expression and Enhanced Sensitivity to Vasoconstrictors

Vasoconstriction and hypertension result from angiotensin II action on the AT₁ receptor and its subsequent activation of second messenger pathways, including PKC. Incubation of endothelial (Chua et al. 1993) and smooth muscle cells (Sung et al. 1994) with angiotensin II stimulates preproendothelin expression via activation of the AT₁ receptor and then PKC. Infusion of angiotensin II for 5 days leads to a marked increase in sensitivity to vasoconstrictors such as phenylephrine, KCl and serotonin, while the sensitivity to endothelin-1 is significantly reduced (Rajagopalan et al. 1997). In vivo treatment with an ET_a receptor antagonist normalized hypercontractile responses and prevented desensitization of the vasculature to endothelin-1. Endothelin expression, as assessed by immu-

nostaining, was increased throughout the vascular wall (Rajagopalan et al. 1997). Superoxide is a strong stimulus for preproendothelin expression in endothelial (Kahler et al. 2000) but also smooth muscle cells (Kahler et al. 2001). Endothelin itself also stimulates vascular $O_2^{\cdot-}$ production (Wedgwood et al. 2001). Increased production of endothelin within smooth muscle and/or endothelial cells may prime PKC, which in turn may mediate hypersensitivity to a variety of vasoconstrictors and activate the NADPH oxidase. Recent studies also indicate that vasoconstriction induced by angiotensin II, but not by endothelin-1, phenylephrine or potassium chloride, is mediated via stimulation of vascular $O_2^{\cdot-}$ production. TEMPOL, a potent SOD mimetic, markedly attenuated the maximal constriction in response to angiotensin II, in spontaneously hypertensive rats (Shastri et al. 2002).

4.5

Role of $O_2^{\cdot-}$ in Angiotensin II-Induced Hypertension

Angiotensin II stimulates vasoconstriction via AT_1 receptor and ROS production, which shorten the half-life of NO. To assess the contribution of $O_2^{\cdot-}$ in angiotensin II-induced hypertension, animals were treated with angiotensin II and the blood pressure lowering effects of liposome encapsulated SOD was tested (Laursen et al. 1997). As a reference constrictor, norepinephrine was used. Despite similar degrees of hypertension, angiotensin II, but not norepinephrine, caused a marked increase in vascular $O_2^{\cdot-}$ production. SOD treatment reduced blood pressure in angiotensin II- but not in norepinephrine-treated rats (Laursen et al. 1997). Likewise, SOD enhanced in vivo hypotensive responses to acetylcholine and in vitro responses to endothelium-dependent vasodilators, indicating that angiotensin II-induced hypertension is likely the result of increased NO degradation, secondary to the stimulatory effects of vascular $O_2^{\cdot-}$ production. To analyze the contribution of vascular NAD(P)H oxidase in angiotensin II-induced hypertension, Fukui et al. studied the time course of angiotensin II-induced hypertension and the expression of the NADPH oxidase subunit p22^{phox}. Blood pressure began to rise within 3 days of angiotensin II treatment, and remained elevated for up to 14 days (Fukui et al. 1997). Expression of p22^{phox} was significantly increased on day 3 and peaked on day 5, after pump implantation. SOD treatment lowered blood pressure and inhibited expression of p22^{phox}, indicating that oxidative stress may be crucial for enzyme expression. Further evidence for the role of $O_2^{\cdot-}$ in the pressor response of angiotensin II was provided by Wang et al. (2002). The authors demonstrate that angiotensin II-induced simultaneously increased blood pressure and blunted vascular $O_2^{\cdot-}$ production in vessels from mice overexpressing human SOD. (Wang et al. 2002).

Similar findings were obtained from animals with renovascular hypertension. In vessels from hypertensive animals, marked endothelial dysfunction was associated with increased NAD(P)H oxidase-mediated superoxide production. In vitro treatment with SOD and the PKC-inhibitor calphostin C, reduced superox-

ide production and improved endothelial function, suggesting an involvement of PKC in activating the oxidase in this particular renin-dependent hypertension (Heitzer et al. 1999).

5 Conclusions

Based on the above findings, we propose that ROS play a pivotal role in mediating endothelial dysfunction under angiotensin II treatment: Angiotensin II activates NAD(P)H oxidases in endothelial and smooth muscle cells and within the adventitia, partly via PKC activation. NAD(P)H oxidase-derived $O_2^{\cdot-}$ may com-

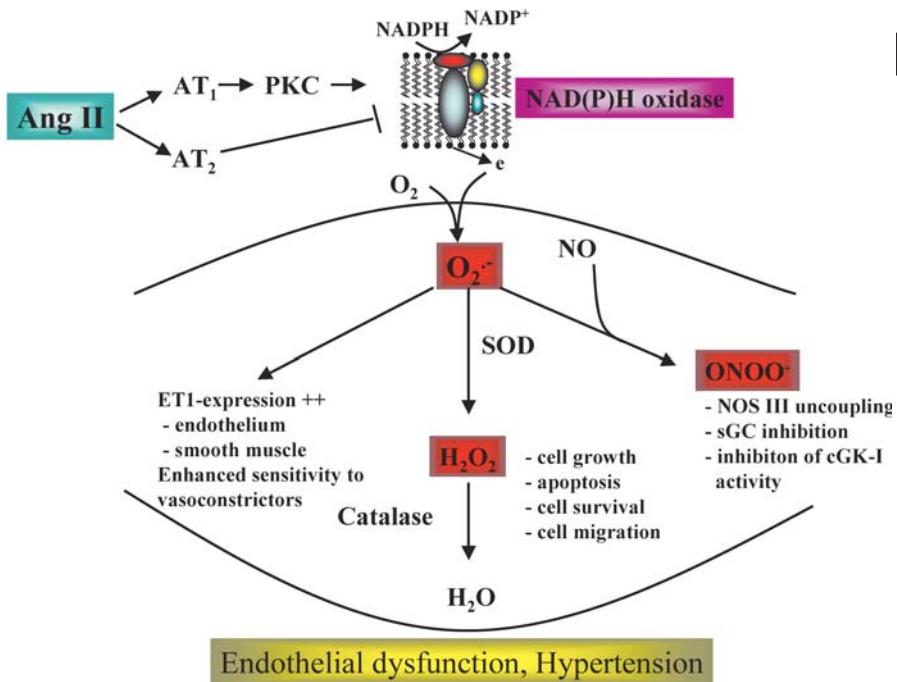


Fig. 1 Scheme depicting mechanisms underlying angiotensin II (*Ang II*)-induced endothelial dysfunction and hypertension. Stimulation of the AT₁ receptor subtype activates NAD(P)H oxidase in a protein kinase C (*PKC*)-dependent fashion. NAD(P)H oxidase activation increases superoxide ($O_2^{\cdot-}$) production within endothelial and smooth muscle cells and within the adventitia, while stimulation of the AT₂ subtype leads to an inhibition of $O_2^{\cdot-}$ formation. Superoxide may react with nitric oxide (*NO*) to produce the reactive intermediate peroxynitrite (*ONOO-*). *ONOO-* may cause NOS III uncoupling, inhibition of the activity of the soluble guanylyl cyclase (*sGC*) and of the cGMP-dependent protein kinase-I. Superoxide dismutase (*SOD*) dismutates $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2), which has been shown to be a potent stimulus for cell proliferation, apoptosis, cell migration and cell survival. Superoxide increases the expression of preproendothelin within endothelial cells and smooth muscle cells, leading to enhanced endothelin-mediated vasoconstriction, but also leading to hypersensitivity of the vasculature to vasoconstricting agonists such as norepinephrine and serotonin. All these events may contribute at least in part to endothelial dysfunction and hypertension in response to angiotensin II treatment

bine with NO to form the highly reactive intermediate ONOO⁻. Peroxynitrite oxidizes the NOS III cofactor BH₄ to BH₂, or may cause oxidation of the zinc-thiolate complex of NOS III. Superoxide may also stimulate and/or inhibit L-arginine methylating or ADMA demethylating enzymes, leading to increased intracellular concentrations of ADMA. Intracellular BH₄ depletion, oxidation of the zinc-thiolate complex of NOS III, and/or increased ADMA concentrations will lead to NOS III uncoupling, which may further increase oxidative stress within vascular tissue. Superoxide also stimulates preendothelin expression in endothelial and smooth muscle cells, leading to enhanced endothelin-mediated constriction and to a hypersensitivity of the vasculature to vasoconstricting agonists such as norepinephrine and serotonin. It is conceivable that all these mechanisms contribute to endothelial dysfunction and hypertension, in response to angiotensin II treatment (see Fig. 1).

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