Free Radical Production and Angiotensin

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Angiotensin II (ANG II) has multiple effects on cardiovascular and renal cells, including vasoconstriction, cell growth, induction of proinflammatory cytokines, and profibrogenic actions. Recent studies provide evidence that ANG II could stimulate intracellular formation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-). This ANG II–mediated ROS formation exhibits different kinetic and lower absolute concentrations than those traditionally observed during the respiratory burst of phagocytic cells, but it likely involves similar membrane-bound NAD(P)H-oxidases. Current evidence suggests that ANG II, through AT_1-receptor activation, upregulates several subunits of this multienzyme complex, resulting in an increase in intracellular O_2^- concentration. ROS are involved in several signal pathways, and redox-sensitive transcriptional factors (AP-1, NF-κB) have been characterized. ANG II–induced ROS play a pivotal role in several pathophysiologic situations of vascular and renal cells such as hypertension, endothelial dysfunction, nitrate tolerance, atherosclerosis, and cellular remodeling. Although these perceptions suggest that drugs interfering with ANG II effects (ACE inhibitors, AT_1 -receptor antagonist) may serve as antioxidants, preventing vascular and renal changes, the clinical studies are not so straightforward. In fact, only specific risk groups, such as patients with diabetes mellitus or renal insufficiency, may benefit from ACE inhibitors, whereas hard endpoints showed no advantage for ACE inhibitors in patients with essential hypertension.

Introduction

More than 100 years ago, Elie Metchnikoff (1845–1916) discovered the defensive role of phagocytes and proposed that these cells constitute a first line of defense in their ability to ingest and digest invading organisms such as bacteria. However, Metchnikoff immediately came under strong attacks by the humoralist school, partly because it was then not well understood how a circulating blood cell should destroy an infective organism rather than spreading the infection through the whole body. It was not until the early 1960s before it became clear that an increase in oxygen consumption occurs during intracellular destruction of microorganisms in neutrophils and macrophages/monocytes, and that reactive oxygen species (ROS) play a pivotal role in this process, now named "respiratory burst." Although it has been known for 50 years that H_2O_2 exists endogenously, the seminal discovery of superoxide dismutase by McCord and Fridovich in 1969 revealed that distinct cellular pathways exist for ROS [1•]. Subsequently, the complex enzymatic mechanism for the intracellular generation of these ROS has been unraveled, and it is now well known that ROS can be generated by various cellular mechanisms involving membrane, cytosolic, and mitochondrial pathways [2]. A large body of evidence has been accumulated over the last two decades. It indicates that ROS, in addition to mediating intracellular killing of pathogens in leukocytes and macrophages, are also important mediators of cell injury under various pathophysiologic conditions [3]. This oxidative stress, leading eventually to tissue injury, was first discovered in the reperfusion phase after organ ischemia, but it is also encountered in a variety of different diseases, ranging from diabetes mellitus to immune-mediated forms of renal disease [3]. Recently, ROS have been recognized as important mediators involved in systems that transduce extracellular signals across the plasma membranes into the cytosol, and may ultimately change transcription of target genes in the nucleus [4]. Angiotensin II (ANG II) is not only a vasoactive peptide involved in hemodynamic regulation, but it has additionally emerged as an important growth and profibrogenic factor in the remodeling of myocardial, vascular, and renal tissues. Recent evidence suggests a link between ANG II and ROS formation in these tissues [5•,6••]. Furthermore, a pertinent relationship between ANG II, ROS, hypertension, and vascular injury has been described [4]. The present review focuses on potential mechanisms of ANG II–induced ROS formation and the consequences of this process for vascular and renal tissues.

What are Reactive Oxygen Species, and Where are They Formed?

The notion that ROS encompass a series of oxygen intermediates including the free radical superoxide anion O_2^- , the nonradical hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl free radical (•OH). A more recently recognized ROS member is singlet oxygen ($^1O_2$), in which one of

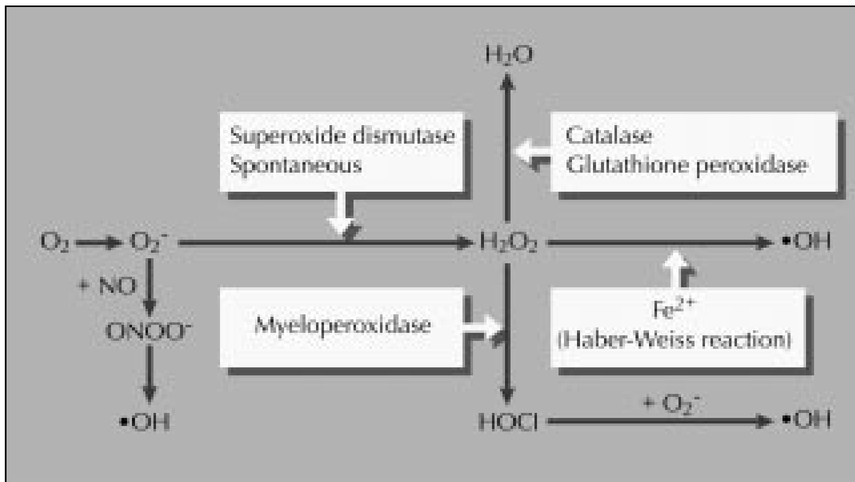

Figure 1. Overview of the generation and metabolism of reactive oxygen species (ROS). The active species are the superoxide anion (O_2^-) and hydroxyl free radical (•OH). Hydrogen peroxide (H_2O_2), a relatively weak oxidant, holds a central position in the further metabolism to other ROS or detoxification to water. The fourth ROS, singlet oxygen ($^1O_2$), is not shown in this overview. NO—nitric acid; Fe^{2+}—iron ion; HOCl—hypochlorous acid.

the electrons is raised to an orbital of higher energy with an inversion of spin [1•]. Some of the pathways for generation and metabolism of ROS are shown in Figure 1. The original source is O_2, which is univalently reduced to form O_2^- by multiple enzymatic pathways (Fig. 2). O_2^- may then spontaneously devolve or be processed by SOD-mediated catalysis into H2O2. This relative weak oxidant holds a central position in the further metabolism to other ROS. H_2O_2 can oxidize chloride to form the reactive hypochlorous acid (HOCl), at least in neutrophils that express the presence of the enzyme myeloperoxidase. HOCl may further react with O_2^- to form the hydroxyl free radical (HOCl + O_2^- → ²OH + O_2 + Cl^-). Alternatively, hypochlorite (OCl-) could further interact with H_2O_2 to produce singlet oxygen (OCl^- + H_2O_2 → $^1O_2$ + H_2O + Cl^-). •OH can be also formed from H_2O_2 and O_2^- by an iron-catalyzed reaction, the so-called Haber-Weiss reaction [2]. However, the role of this reaction in vivo has been questioned because of the limited availability of free iron, which normally binds to lactoferrin. Interestingly, the Haber-Weiss reaction is also implicated in the generation of singlet oxygen ($^1O_2$). Lastly, nitric oxide (NO) scavenges O_2^- yielding peroxynitrite ($ONOO^-$), which may decompose into nitrate and •OH.

Due to 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 (Fig. 1). Since H_2O_2 is less reactive than O_2^- superoxide dismutase, which is actually a whole family of several homodimeric metalloenzymes, may be considered part of a detoxification pathway neutralizing superoxide anions. Furthermore, H_2O_2 is reduced by catalase or gluthathione peroxidase to H_2O. In particular, tetrameric gluthathione peroxidase serves as a detoxification pathway for several noxious lipid peroxides.

The key initial step in formation of all ROS is the conversion of molecular oxygen (O_2) into the superoxide anion (O_2^-). Several enzymatic pathways can generate O_2^- (Fig. 2), but in quantitative terms, the electron transport chain in mitochondria is the most important source. Other pathways may represent leaks that allow electrons

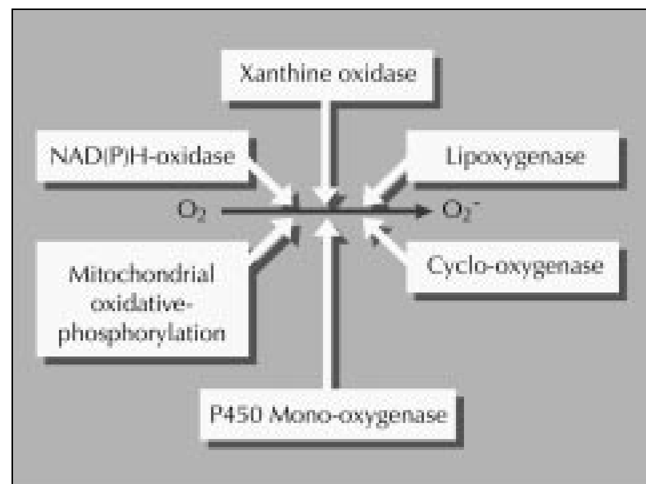

Figure 2. Many different cellular enzymes catalyze the generation of superoxide anion from molecular oxygen. The membrane-bound NAD(P)H-oxidase is essential for angiotensin II–mediated O_2^- generation.

to reach O_2 outside of the controlled mitochondrial environment. The xanthine-oxidase system plays a pivotal role in the formation of O_2^- from the ATP breakdown product hypoxanthine during reperfusion injury after prolonged ischemia.

The NAD(P)H-oxidase is the enzymatic complex responsible for the generation of O_2^- in phagocytes during the respiratory burst [4]. In addition, a membrane-bound NAD(P)H-oxidase system is also present in many nonphagocytic cells, including endothelial and vascular smooth muscles cells (VSMC) and mesangial cells, podocytes, and proximal tubules in the kidney. Under normal conditions, the NAD(P)H-oxidase is dormant in nonactivated neutrophils, with only two subunits, glycoprotein (gp)91phox (for phagocyte oxidase) and p22phox, constituting the membrane-bound cytochrome b_{558} (Fig. 3). The flavoprotein FAD is a cofactor linking NADPH and cytochrome b_{558}. 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

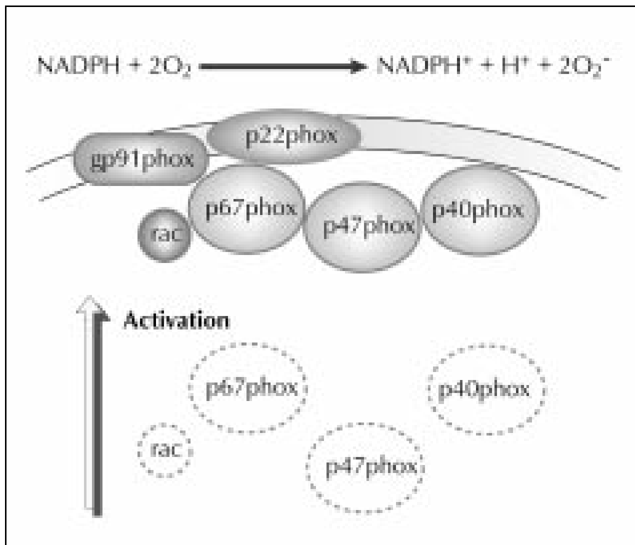

Figure 3. Overview of the multienzyme complex of neutrophil NAD(P)H-oxidase. The subunits rac, p67phox, p47phox, and p40phox reside under normal conditions in the cytosol and associate with the membrane-bound gp91phox/p22phox subunits only after activation. Although differences exist between this depicted multienzyme from neutrophils and the NAD(P)H-oxidase from nonphagocytic cells, there are several common subunits, including p22phox, that play an important role in ANG II–mediated reactive oxygen species generation. ANG II stimulates transcription of p22phox subunits in various cells, providing one mechanism for how the vasopeptide may activate NAD(P)H-oxidase.

trigger electron transport [4]. Rac2 exhibits a high affinity for cytochrome b_{558} and appears to be constitutively associated with the cell membrane. On cellular activation inducing respiratory burst, the additional components p67phox, p47phox, p40phox, and rac1 shift from the cytosol to the membrane. These proteins bind to the poly-L-proline-rich domain of p22phox through the interaction of src homology domain-3 (SH_3). Further SH_3-mediated interaction associates p67phox with p47phox. Mutations in each of these NAD(P)H-oxidase subunits have been described, resulting in attenuated ROS production with the clinical phenotype of chronic granulomatous disease, a rare disorder with increased susceptibility to bacterial and fungal infections.

Although the detailed structure of nonphagocytic NAD(P)H-oxidase is only incompletely understood, it nevertheless shares several characteristics with its neutrophil counterpart, including sensitivity to the flavoprotein inhibitor diphenylene iodinium (DIP) [5•,6••]. However, compared to phagocytes in which O_2^- generation during the respiratory burst is fast and massive with release of ROS into the extracellular environment, ROS formation in nonphagocytic cells is principally restricted to intracellular space and occurs over a period of hours with a quantitatively decreased magnitude. Some important structural differences may exist between the neutrophil and nonphagocytic NAD(P)H-oxidases, and the latter apparently express a large subunit, not similar to gp91phox, of neutrophils [4]. Furthermore, p22phox has been cloned from VSMC, is abundant in these cells, and the NAD(P)H-oxidase-mediated ROS generation in VSMC and renal proximal tubular cells are diminished by p22phox antisense oligonucleotides, indicating an important role of this subunit in holoenzyme function [5•,6••]. Finally, the nonphagocytic enzymes may preferentially use more NADH than NAD(P)H as coenzyme.

Reactive Oxygen Species as Intracellular Signal Transduction Systems and Modulators of Transcriptional Pathways

In striking contrast to phagocytic cells in which ROS produced during the respiratory burst destroy microorganisms, these species may function as signal transduction intermediates in other cells. As shown more than a decade ago, exogenous maneuvers to increase ROS such as xanthine/xanthine oxidase or H_2O_2 lead to the induction of immediate early genes including *c-jun*, *c-fos*, and *c-myc*, as well as stimulate proliferation of mouse epidermal cells and fibroblasts [7,8]. Similar effects were subsequently found in VSMC and in other cardiovascular and renal cell types [9–11]. Members of the *c-jun* and *c-fos* families interact through a leucine zipper to generate homo- and heterodimers that are capable of binding to AP-1 regions in target genes [12]. Indeed, AP-1 is now among the most well studied transcriptional factors influenced by the cellular redox state [12,13]. Meanwhile, ROS-induced gene transcription mediated through AP-1 has been demonstrated for many genes playing a role in cardiovascular pathology including adhesion molecules, proinflammatory chemokines (MCP-1), and growth stimulatory and apoptotic genes [13–16]. However, NF-κB was the first transcription factor shown to be activated by oxidative stress [14]. This conclusion was reached on experiments demonstrating that antioxidants inhibit NF-κB activation. NF-κB is a prototype of a whole family of transcription factors that are retained in the cytosol as heterodimers in an inactive form [14]. This dimer is composed of various members of the NF-κB/Rel family, which binds an inhibitor protein called IκB. Recent evidence suggests the existence of multiple forms of IκB. Upon activation, IκB is phosphorylated, degraded, and NF-κB is released and moves into the nucleus to bind to target DNA elements and activates transcription of these genes [13].

The activation of AP-1 and NF-κB transcription factors through oxidative stress can be explained by findings that ROS affect multiple signal transduction cascades upstream of these transcription factors [17]. Endothelial and VSMC challenged with H_2O_2 or xanthine/xanthine oxidase respond with transient release of Ca^{2+} from intracellular stores [17,18]. ROS-mediated inhibition of ATP-dependent Ca^{2+} pumps may be a potential mechanism for this intracellular increase in Ca^{2+} [19]. Bass and Berk showed in 1995 that a superoxide-generating agent activates mitogen-activated protein kinases (MAPKs) in VSMC [20]. In addition,

ROS-induced activation of src, SAPK/JNK, and p38 kinase pathways, as well as inhibition of protein phosphatases, have been described in various cell types [21•,22].

Many of these ROS-stimulated signal transduction pathways and activation of transcription factors have been elucidated using xanthine/xanthine oxidase or H_2O_2 as an exogenous source for the formation of ROS [23,24]. A more recent conception is that ROS are intracellularly formed after exposure of cells to growth factors and cytokine [22,25•]. Membrane-bound NAD(P)H-oxidase is likely responsible for this induction of ROS [26••,27–29]. Relatively low concentrations of O_2^- may, in turn, interact with many of the signal transduction pathways as intermediates of normal signal transduction pathways, rather than as pathophysiologic alterations forced onto cells by an exogenous source of ROS or the respiratory burst. For example, Irani *et al.* demonstrated an important role for O_2^- in ras-induced cell cycle proliferation in fibroblast, independent of MAPK [26••,27].

Angiotensin II and Reactive Oxygen Species

The first evidence of ANG II–mediated ROS production came 10 years ago from a single author [30]. Wilson performed acute ANG II infusion experiments into Wistar rats in the presence or absence of different free radical scavengers including superoxide dismutase (SOD), catalase, dimethyl sulfoxide [30]. These scavengers did not reduce acute ANG II–induced hypertension, they partly inhibited vascular hyperpermeability and cellular damage. Although Wilson did not directly measure ROS, he straightforwardly suggested that ANG II induces ROS formation in this system [30]. Subsequent in vitro studies demonstrated that treatment of cultured VSMC with ANG II for 4 to 6 hours increased intracellular O_2^- as measured by lucigenin assay [31]. This ANG II-stimulated O_2^- production was transduced through AT_1-receptor and was caused by activation of membrane-bound NAD(P)H-oxidase because DIP and p22phox antisense oligonucleotides attenuated this response [31]. Furthermore, ANG II–mediated production is followed by an increase in intracellular H_2O_2 by endogenous SOD present in VSMC [32]. AT_1-receptor transduced ROS formation, depending on NAD(P)H-oxidase, has been also described in the kidney in cultured mesangial and proximal tubular cells, podocytes as well as in human macrophages [6••,33,34]. Immunohistochemical studies in rabbit aortic sections revealed the presence of p22phox, gp91phox, p47phox, and p67phox localized exclusively in the adventitia [35•]. Cultured fibroblasts, isolated from the adventitia of rabbits, increased production of ROS after challenge with ANG II [35•]. Infusion of ANG II into rats for 5 days increased blood pressure and doubled vascular generation [36••]. This increase in ROS formation was mediated by AT_1- receptors [36••]. Further experiments in vascular homogenates revealed that the oxidase activated by ANG II in vivo was membrane bound and was stimulated by NADH to a greater extent than NAD(P)H [36••,37].

Similar observations have been made in rat aortas when the endogenous renin-angiotensin system was stimulated, using the two kidney-one clip (2K-1C) hypertension model [38]. In these hypertensive rats, endothelium relaxation was impaired, and vascular O_2^- formation was significantly increased compared with controls [38]. Pharmacologic inhibitor studies of vascular homogenates from 2K-1C animals demonstrated that the major source of $O2^-$ was a NAD(P)H-oxidase that was activated by a protein kinase C-dependent mechanism [38]. The first in vivo evidence in humans of a relationship between ANG II and ROS was provided by Dijkhorts-Oei *et al.*, who infused ANG II into the brachial artery of healthy volunteers [39•]. ANG II–induced vasoconstriction was significantly attenuated by vitamin C co-infusion, suggesting that ROS contributed to the vasoconstriction [39•].

The mechanisms of how ANG II activates NAD(P)H-oxidase are not well understood. It is clear that the response is mediated by AT_1-receptors and involves protein kinase C in some systems [4,36••,38]. ANG II stimulates p22phox transcription in VSMC, rat aortas, and renal proximal tubular cells [5•,6••,40]. This increase in p22phox mRNA expression was accompanied by an increase in NAD(P)H-oxidase activity [5•,6••,40]. Further evidence for an important role of p22hox in ANG II–mediated O_2^- generation emanated from antisense experiments interfering with p22phox expression [5•,6••]. ANG II also stimulates the transcription of p67phox in rabbit aortic adventitial fibroblast [41•]. Thus, ANG II apparently increases NAD(P)H-oxidase by stimulating synthesis of some of its subunits. However, the exact signal transduction pathways are only incompletely understood. Furthermore, the fate of these newly synthesized subunits remains unclear after ANG II is withdrawn because O_2^- generation declines in the absence of the vasopeptide. Perhaps these subunits will be intracellularly degraded by proteases after dissociation from the NAD(P)H-oxidase multienzyme complex, but this hypothesis needs further experimental confirmation.

Pathophysiologic Effects of Angiotensin II–induced Reactive Oxygen Species

Pathophysiologic consequences of ANG II–mediated ROS formation are depicted in Figure 4 and include endothelial dysfunction that may be arbitrarily defined as a reduced vasodilatation in the presence of acetylcholine [42]. This endothelial dysfunction is mainly due to a decrease in local NO synthesis and occurs in clinical situations such as hypercholesterolemia, diabetes mellitus, hypertension, and smoking [42]. In many situations, NO has opposite effects from ROS, including vasodilatation, inhibition of platelet adherence and aggregation, suppression of proinflammatory cytokines, and growth suppression [42]. Although there is a complex interaction between ANG II, ROS, and induction of NO-synthase,

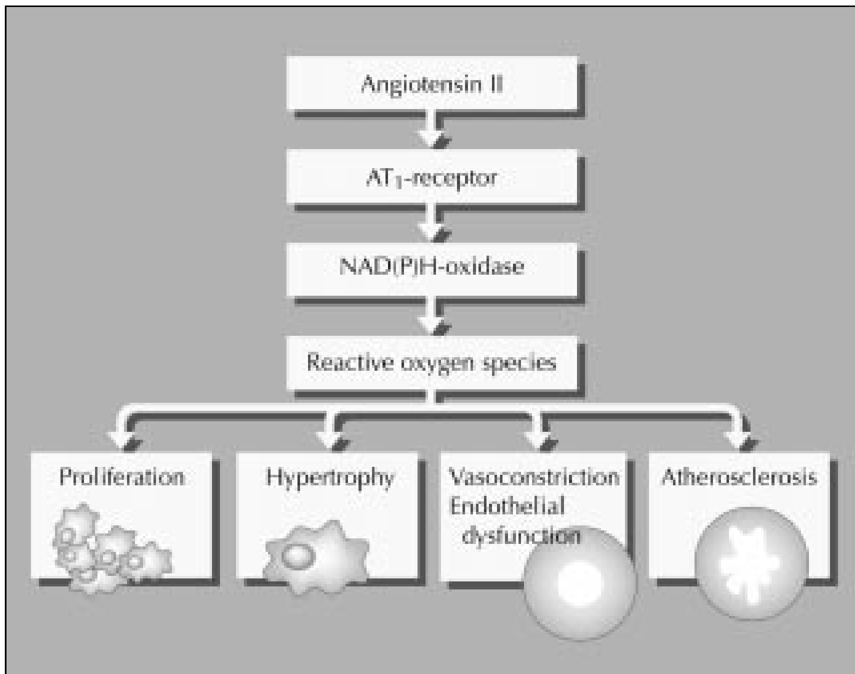

Figure 4. Pathophysiologic effects of angiotensin II–mediated reactive oxygen species (ROS) formation.

accumulating evidence suggest that O_2^- interacts with NO, resulting in the destruction of NO associated with the formation of highly active •OH after generation of peroxynitrite as intermediate [42]. Münzel *et al.* were the first who proposed that this mechanism underlies nitrate tolerance in which nitroglycerin-derived NO is degraded by O_2^-, particularly if ROS formation is stimulated in the presence of high circulating or local ANG II [43]. Inducible NO generation in cultured proximal tubular cells is attenuated in the presence of ANG II without any change in transcription of inducible NO-synthase, suggesting that O_2^--mediated NO neutralization may also play a role in renal cells [45].

ANG II-stimulated O_2^- formation directly contributes to hypertension, likely via degradation of endothelium-derived NO, because treatment of ANG II-infused rats with liposome-encapsulated SOD reduced blood pressure by 50 mm Hg, but had no effect on hypertension induced by norepinephrine infusion [37]. ANG II-infusion increased transcription of extracellular SOD (ecSOD) in mice, independent of concomitant hypertension. Interestingly, ANG II-induce hypertension is more severe in rats (a species lacking ecSOD) than in mice, indicating that this increase in ecSOD may represent an important compensatory mechanism, partly blunting the ROS-mediated increase in blood pressure [45•]. An increase in ROS formation, likely induced by ANG II, is essential for uremic hypertension in a rat model with 5/6 nephrectomy [46]. Polymorphonuclear leukocytes from patients with essential hypertension exhibit an increase in ROS production [47]. However, whether ANG II is involved in this process remains unclear.

ANG II–induced ROS formation may also play an important role in the development of atherosclerosis [48]. Warnholtz *et al.* demonstrated in hypercholesteremic rabbits an AT_1-receptor up-regulation leading to an increased NAD(P)H-dependent vascular production [48]. An AT_1-receptor antagonist improved endothelial dysfunction and reduction of early plaque formation, suggesting an important role for ANG II–mediated ROS formation in this model [48]. ANG II–mediated oxidation of low-density lipoprotein (oxLDL) and an unpregulation of LOX-1, the endothelial receptor for OxLDL, may contribute to the relationship between ANG II and ROS in the pathogenesis of atherosclerosis [49•]. ANG II is also a well-characterized growth factor for vascular cells. Similarly, it has been shown that ANG II–induced O_2^- formation is essential for hypertrophy of VSMC [4,5•]. ANG II stimulates hypertrophy of proximal tubular cells and O_2^- ,as a second messenger, causes this cell cycle arrest in the G_1-phase by inducing $p27^{Kip1}$, an inhibitor of cyclin-cyclin-dependent kinase complexes [6••].

Clinical Consequences?

Although the perception of ANG II–mediated ROS formation and its deleterious consequences suggest that drugs blocking the renin-angiotensin system (*eg,* ACE inhibitors, AT_1-receptor antagonists) may serve as antioxidants, preventing vascular and renal injuries, the clinical studies are not as straightforward as the culture dish or the metabolic cage. ACE inhibitor treatment has undoubtedly been shown to be beneficial for patients with specific diseases such as heart failure and diabetes mellitus. Furthermore, therapy with an ACE inhibitor is currently the only known treatment to prevent the progression of chronic renal diseases. However, the situation for patients with essential hypertension is less clear. In fact, two trials showed no advantage to treatment with ACE inhibitors over conven-

tional therapy in patients with hypertension [50,51]. After the initial brouhaha following the publication of the ELITE 1 study, the recent observation from the ELITE 2 trial, that there is no advantage to using an AT_1-receptor antagonist compared to an ACE inhibitor in patients with heart failure, as well as data showing an independent role for aldosterone [52], clearly demonstrate that ANG II is conceivably more complex than we are accustomed to think. The relationship between ANG II, AT_1-receptor activation, and ROS formation, leading finally to vascular and renal injury, is certainly not a simple one. Prospective randomized trials are necessary to establish that antagonizing ANG II action will reduce ROS generation in humans.

Conclusions

In vivo and in vitro studies provide ample evidence that ANG II could stimulate intracellular formation of O_2^- by upregulating subunits of the membrane-bound NAD(P)H-oxidase in vascular and renal cells. O_2^- is involved in several signal pathways and redox-sensitive transcriptional factors, including AP-1 and NF-κB, suggesting that O_2^- is an important second messenger of the transcriptional effects of ANG II. ANG II–induced O_2^- may play a pivotal role in several pathophysiologic situations involving vascular and renal tissue, such as hypertension, endothelial dysfunction, nitrate tolerance, atherosclerosis, and cellular remodeling. Although this concept suggests that ACE inhibitors may exert antioxidative effects in vivo, this theoretical benefit has not been translated into clinical superiority of this class of drugs compared with conventional therapy in patients with essential hypertension.

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