Role of Superoxide in Modulating the Renal Effects of Angiotensin II

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Abstract—Angiotensin II is known to stimulate NADPH oxidase–dependent superoxide (O$_2^-$) generation, which may contribute to the acute renal vasoconstrictor and antinatriuretic actions of this peptide. To evaluate this hypothesis, the effects of a superoxide dismutase mimetic (tempol) or a NADPH inhibitor (apocynin) on the angiotensin renal actions were studied. Renal cortical nitric oxide (NO) was measured electrochemically in vivo. Tempol increased sodium excretion and NO levels. Apocynin raised renal blood flow, glomerular filtration rate, sodium excretion, and NO levels. These results indicate the presence of an endogenous NADPH oxidase–dependent O$_2^-$ generation that may modulate renal function by scavenging NO. Angiotensin II infusion reduced renal blood flow, glomerular filtration, sodium excretion, and NO levels in a dose-dependent manner. The angiotensin receptor antagonist valsartan, tempol, or apocynin blunted the angiotensin effects on renal excretion and NO, suggesting that angiotensin receptors stimulation induces the NADPH oxidase–dependent O$_2^-$ generation that might reduce NO bioavailability. This idea is supported by the finding that angiotensin increased O$_2^-$ generation in renal homogenates, and this effect was prevented by valsartan, apocynin, or tempol. These results indicate that some of the acute renal effects of angiotensin II may be enhanced by an increased NADPH oxidase–derived O$_2^-$ production that reduces renal NO bioavailability. (Hypertension. 2003;42:1150-1156.)

Key Words: antioxidants ■ kidney ■ oxygen ■ nitric oxide ■ angiotensin II

The renin-angiotensin system plays a critical role in the regulation of renal function and arterial pressure. In addition to the classic signal transduction mechanisms, the angiotensin II (Ang II) receptor type 1 (AT$_1$R) activates the NADPH oxidase–dependent generation of superoxide (O$_2^-$). This effect is produced primarily in vascular smooth muscle cells (VSMCs) and fibroblasts. The major enzyme responsible for vascular O$_2^-$ appears to be NADPH oxidase, which catalyzes the production of O$_2^-$ by the reduction of oxygen. O$_2^-$ is known to avidly react with nitric oxide (NO) to form peroxynitrite, thus diminishing the half-life of NO. Ang II regulates and increases NADPH oxidase–dependent O$_2^-$ production in cultured VSMCs, in aortic adventitial fibroblasts, and in mesangial cells.

A role for O$_2^-$ in the control of renal function seems likely because recent studies in rats indicate that O$_2^-$ production in the renal medulla exerts vasoconstrictor as well as antidiuretic and antinatriuretic actions. The mRNA encoding the $\delta$ subunit of the classic phagocyte NADPH oxidase and the proteins p22phox, p47phox, and p67phox have been shown to be present in the normal adult rat kidney. It has been reported that treatment with an AT$_1$R antagonist, a superoxide dismutase mimetic, or a NADPH oxidase inhibitor reduces the oxidative stress in spontaneously hypertensive rats, inhibits the enhanced tubuloglomerular feedback, blunts sodium reabsorption in the thick ascending limb of the loop of Henle, and decreases the NADPH oxidase–stimulated O$_2^-$ production in rat arteries. In addition, the intrarenal infusion of a superoxide dismutase inhibitor produces marked vasoconstriction and antinatriuresis; because these effects are potentiated by NO synthesis inhibition, it has been postulated that the renal actions of superoxide are buffered by nitric oxide. Therefore, it seems that the balance between superoxide and nitric oxide is important in controlling renal function, but the in vivo effects of Ang II affecting this equilibrium and contributing to modulate renal excretion and hemodynamics are unknown.

Although the experimental evidence supports a role for O$_2^-$ in the regulation of renal function, little is known about the renal effects of NADPH oxidase inhibitors or O$_2^-$ scavengers in vivo. In addition, the contribution of NADPH oxidase–dependent generation of O$_2^-$ to the acute renal actions of Ang II remains to be established. In the present study, the response...
to intrarenal infusions of a NADPH oxidase inhibitor (apocynin) and a SOD mimic (tempol) were evaluated to exclude that sources of superoxide other than NADPH oxidase may regulate renal function. Furthermore, the effects of pretreating the kidney with tempol or apocynin on the renal actions of Ang II were studied.

Methods

Experiments were performed on 63 anesthetized male Sprague-Dawley rats (weight, 250 g) anesthetized with ketamine (30 mg/kg IM) and thiopental (50 mg/kg IP). The surgery and basic experimental techniques were previously described.16 The left kidney was exposed and stabilized in a renal cup to allow for measurement of NO, bathed in saline at 37°C. Renal cortical [NO] was measured with carbon fiber microelectrodes17 by differential normal pulse voltammetry. With this technique, NO produces an oxidation peak at 0.65 V.18

All drugs (tempol, apocynin, valsartan, and Ang II) were administered intravenously16 in the same volume of fluid (isotonic NaCl solution, at a rate of 50 μL/min), and an intravenous infusion of saline containing [3H]inulin (1 μCi/mL, to allow for glomerular filtration rate measurements) at a rate of 1 mL/100 g per hour was given. The lucigenin-derived chemiluminescence assay3 was used to determine superoxide in renal cortex homogenates.

Protocol 1

Protocol 1 involved characterization of the response of the NO electrodes in vivo (n=5). L-Nitroarginine methyl ester (10 mg/kg) was given intravenously, and the NO peak current was measured. An intravenous infusion of sodium nitroprusside was started at a dose (20 μg/kg per minute) that normalizes arterial pressure, and the NO current was re-determined.

Protocol 2

Protocol 2 involved time course studies (n=7). Renal function and [NO] levels were evaluated during a 1-hour period.

Protocols 3 and 4

Protocols 3 and 4 involved characterization of the renal effects of tempol or apocynin (n=6). After a control period, increasing doses of tempol (4, 8, and 16 μmol/kg per minute to yield 250, 500, and 1000 μmol/L in renal arterial plasma at baseline renal blood flow [RBF]19–22 or apocynin (5, 10, and 50 μmol/kg per minute to achieve 300, 600, and 3000 μmol/L renal arterial plasmatic concentrations at baseline RBF19–22) were consecutively infused into the renal artery for 10 minutes each, and renal function and [NO] were measured during a 10-minute clearance period for each dose. The highest dose of tempol or apocynin tended to decrease arterial pressure; this was avoided by occluding the mesenteric and celiac arteries and by using an aortic clamp.20 Apocynin is a specific inhibitor of NADPH oxidase in blood vessels, with no other known actions.14

Protocol 5

Protocol 5 involved the renal effects of tempol plus apocynin (n=6). These experiments evaluated whether these compounds have additive effects, as an indication that they might produce actions other than suppressing O2-. The doses were the same used above.

Protocols 6 to 9

Protocols 6 to 9 involved the renal effects of Ang II alone (n=7) or in rats pretreated with valsartan (n=7), tempol (n=7), or apocynin (n=6). Either saline, valsartan (160 nmol/kg per minute to yield ~10 μmol/L in renal arterial plasma), tempol (8 μmol/kg per minute to achieve 500 μmol/L in renal arterial plasma), or apocynin (10 μmol/kg per minute to obtain 600 μmol/L in renal arterial plasma) were infused. The doses of tempol or apocynin used were the maximum that alter renal function without affecting arterial pressure.

Then, Ang II at doses of 1.6, 16, and 160 pmol/kg per minute was successively infused intrarenally (to obtain 0.1, 1, and 10 nmol/L renal arterial plasma concentrations at baseline RBF).

Protocol 10

Protocol 10 involved superoxide levels in renal cortex homogenates (n=6). Ang II (10 nmol/L), valsartan (10 μmol/L), apocynin (600 μmol/L), tempol (500 μmol/L), Ang II plus valsartan, Ang II plus tempol, or Ang II plus apocynin (same doses) were added to the samples and incubated in presence of lucigenin.

Results

Baseline values of renal function and [NO] levels in all groups are presented in the Table.

Characterization of the Response of the NO Electrode In Vivo

When the electrode is introduced in the renal cortex, an oxidation current peak can be measured at 650 mV (Figure 1). This voltage coincides with the oxidation peak found in vitro in anaerobic NO solutions. The peak height decreases after L-NAME is given (to 24% of control) and is restored during the infusion of the NO donor sodium nitroprusside (to 93% of control), indicating that it is probably due to NO.

Renal Effects of Tempol, Apocynin, or Tempol Plus Apocynin

Tempol had no significant effects on RBF and slightly decreased glomerular filtration rate (GFR) (−13%) and the filtration fraction (FF, −15%) at the higher dose used (Figure
2). On the other hand, apocynin increased RBF (+31%) and GFR (+19%), having no effect on FF. The coadministration of tempol plus apocynin had no additive effects on renal hemodynamics.

Tempol raised \( U_{\text{Na}} \) (+33%) and \( \text{FENa} \) (31%, Figure 3). Apocynin also increased \( U_{\text{Na}} \) (+122%) and \( \text{FENa} \) (+67%). The coadministration of tempol plus apocynin had no additive effects on renal sodium excretion.

Tempol increased [NO] (88%) and \( U_{\text{NO}} \) (+68%, Figure 4). In addition, apocynin raised [NO] (+114%) and \( U_{\text{NO}} \) (+98%). The infusion of tempol plus apocynin had no additive effects on renal [NO].

### Renal Effects of Ang II, Tempol Plus Ang II, and Apocynin Plus Ang II

Ang II similarly reduced RBF (-36%) and GFR (-35%), having no effect on FF (Figure 5). During the infusion of tempol or apocynin, Ang II still reduced RBF, but it had no effect on GFR, thus increasing FF (Figure 5).

Ang II lowered \( U_{\text{Na}} \) mainly because of the fall in filtered load, because it did not decrease \( \text{FENa} \) (Figures 5 and 6). Pretreatment with tempol blocked the antinatriuresis produced by Ang II by preventing the fall in GFR, with no effects on tubular sodium reabsorption. In rats given apocynin, however, Ang II increased \( U_{\text{Na}} \) (+61%) by decreasing tubular sodium reabsorption (Figure 6).

### Renal Effects of Ang II in Rats Pretreated With Valsartan

Valsartan lowered mean arterial pressure (MAP) (from 120±3 to 114±3 mm Hg) and raised RBF (from 7.67±0.31 to 8.73±0.40 mL/min per gram), but it had no effect on GFR, \( U_{\text{Na}} \), and [NO]. The subsequent intrarenal infusion of Ang II during the administration of valsartan had no effects on MAP, RBF, GFR, and [NO]. The actions of Ang II on \( U_{\text{Na}} \) were blunted by valsartan, because only the highest dose of angiotensin used (160 pmol/kg per minute) decreased \( U_{\text{Na}} \) (from 7.09±0.67 to 6.08±0.64 Eq/min per gram).

### \( O_2^- \) Levels in Renal Cortex Homogenates

The levels of \( O_2^- \) in renal homogenates, as estimated by lucigenin-enhanced chemiluminescence, increased by +17% in Ang II–incubated (10 nmol/L) cortical homogenates compared with basal \( O_2^- \) generation (2283.94±188.21 photons/min per milligram of protein) (Figure 8). In contrast, valsar-
tan (10 μmol/L), tempol (500 μmol/L), or apocynin (600 μmol/L) markedly reduced basal $O_2^-$ levels (by −37%, −30%, and −60%, respectively). In renal homogenates preincubated with either valsartan, tempol, or apocynin, Ang II had no effect on $O_2^-$ levels.

**Discussion**

The present study examined the renal responses to an intra-renal infusion of either a SOD mimetic or a NADPH oxidase inhibitor. Scavenging renal superoxide with tempol increased sodium excretion as well as renal cortex NO activity and nitrite plus nitrate excretion. In addition, the inhibition of NADPH oxidase with apocynin raised RBF, GFR, UNaV, [NO], and UNOxV. The fact that these two compounds have no additive effects is compatible with the hypothesis that both affect renal function by reducing superoxide. These results indicate the existence of a basal $O_2^-$ production in renal tissue, which appears to be NADPH oxidase–dependent, exerting a tonic regulatory action on renal hemodynamics and tubular sodium transport that may be partly mediated by affecting renal NO bioavailability. This physiological mechanism is compatible with the recent finding that inhibition of renal superoxide dismutase induces renal vasoconstriction and reduces urinary sodium excretion. Interestingly, tempol produced less renal vasoconstriction and natriuresis than apocynin. The reasons explaining this interesting phenomenon are unknown, but one may speculate that it may be related to the fact that tempol produces hydrogen peroxide from superoxide, and it might be responsible for decreasing GFR and limiting the vasodilation and increased natriuresis produced by NO during the infusion of tempol, as it has been recently shown. Also, because apocynin affects renal function more than tempol, it can be concluded that NADPH oxidase appears to be the only source of superoxide influencing renal function. An alternative explanation would be that the doses of tempol used were too low to affect renal function. This seems unlikely, because the concentrations reached in renal arterial plasma are in the range previously tested in vitro. In any case, higher doses of tempol cause arterial pressure to drop, and therefore their effect on renal function cannot be evaluated in vivo.

When the rats were infused intrarenally with increasing doses of Ang II, RBF, GFR, UNaV, [NO], and UNOxV fell in a dose-dependent manner, as described previously in dogs and rats. Ang II increases filtration fraction in dogs, and this has been interpreted as a preferential efferent arteriolar action; however, this effect does not take place in rats because the peptide causes in this species similar afferent and efferent vasoconstriction. The generation of $O_2^-$ induced by Ang II may contribute to the renal vasoconstriction and antinatriuresis either by a direct effect on vascular smooth muscle cells or indirectly, by acting on the juxtaglomerular apparatus and enhancing the tubuloglomerular feedback response. The Ang II–induced decrease in [NO] observed in the present study is consistent with previous reports that a chronic infusion of Ang II markedly depressed vascular NO production (despite the fact that Ang II increased NOS protein in the kidney) by inducing NADPH oxidase–dep-
dent \(O_2\) generation and lowered urinary NOx excretion. This interaction between \(O_2\) and NO has been demonstrated in vitro because it has been shown that \(O_2\) decreases NO bioavailability in single renal tubules. However, these findings contrast with other reports suggesting that Ang II increases NO concentrations in the renal cortex and that activation of the renin-angiotensin system during sodium depletion, or Ang II infusion in rats maintained on a normal sodium intake, stimulate a vasodilator signaling cascade that includes bradykinin, NO, and cGMP. The reasons explaining these differences are unknown, but they may be related to the fact that in these studies Ang II was infused intravenously, and the concentration of the peptide reaching the kidney may have been insufficient to stimulate \(O_2\) generation.

The acute intrarenal infusion of valsartan lowered MAP and raised RBF without affecting GFR, \(U_{NaV}\), or [NO]. Similarly, it has been reported that losartan had no effect on renal cortical cGMP levels in sodium-depleted or sodium-replete rats. In addition, the effects of Ang II on RBF, GFR, \(U_{NaV}\), [NO], and \(U_{NOxV}\) were totally abolished by pretreatment with the \(AT_R\) antagonist. These results indicate that the physiological effects of Ang II are mainly mediated through the stimulation of the \(AT_R\). The fact that the fall observed in cortical NO concentration and \(U_{NOxV}\) is prevented by valsartan suggests that the increase in renal \(O_2\) levels is triggered by \(AT_R\) stimulation, as previously suggested. An alternative possibility would be that Ang II may act on \(AT_2\) receptors during \(AT_R\) blockade and increase NO levels.

Pretreatment with tempol had no effect on the renal vasoconstriction produced by Ang II. However, the SOD mimetic prevented the Ang II–induced fall in GFR, therefore increasing filtration fraction, suggesting a preferential afferent dilatory effect. Tempol also maintained renal cortical NO, \(U_{NOxV}\), and \(U_{NaV}\) during the infusion of Ang II. These results are compatible with the idea that the increase in \(O_2\) generation produced by Ang II reduces NO within the kidney, thus contributing to some of the excretory and glomerular effects of the peptide. It is known that Ang II reduces glomerular surface area by promoting mesangial cells contraction and \(O_2\) production. It is also known that those mesangial effects of Ang II can be blunted by NO. Therefore, it seems reasonable to postulate that the effect of tempol preventing the fall in GFR produced by Ang II may be due to increased NO bioavailability, as previously suggested. A role for \(O_2\) in potentiating the antinatriuresis produced by Ang II seems likely, since it is known that \(O_2\) reduces sodium excretion and because this Ang II effect is also prevented by tempol. In addition, tempol precludes the increase in plasma endothelin and isoprostanes caused by Ang II, and this action is thought to participate in impairing some of the renal hemodynamic Ang II effects.
In the present study, pretreatment with the NADPH oxidase inhibitor apocynin raised RBF, GFR, U Na V, [NO] and increased filtration fraction, indicating a preferential afferent arteriolar effect. This suggests that the stimulation of NADPH oxidase–dependent O2•− generation contributes to the vasoconstrictor effects of Ang II and is compatible with the recent finding that O2•− is a potent renal vasoconstrictor.9,15 Apocynin abolished the actions of Ang II on GFR, U Na V, [NO], and U NOx V. It has been reported that Ang II specifically activates the NADPH oxidase enzyme system and promotes O2•− generation.6 Also, it is known that apocynin blocks the activation of NADPH oxidase12 and is capable of increasing NO bioavailability.14 The five subunits of the classic phagocyte NADPH oxidase are expressed in the normal adult rat kidney at specific sites such as the renal artery, afferent and efferent arterioles, glomeruli, distal tubules, and macula densa.16 This suggests that the NADPH oxidase–dependent increased renal O2•− generation stimulated by Ang II may limit NO bioavailability in the kidney. Therefore, O2•− appears to participate as a modulator in the Ang II regulation of Na+ transport and renal hemodynamics.

To further evaluate the idea that Ang II increases renal NADPH oxidase–induced O2•− generation and reduces [NO], we measured the O2•− levels in renal cortex homogenates incubated with Ang II, valsartan, tempol, or apocynin. We found that Ang II enhanced O2•− production and that this effect was blocked by coincubation with valsartan, tempol, or apocynin.14,34 In addition, the basal levels of O2•− were diminished by valsartan, tempol, and apocynin. These in vitro experiments indicate that there is a basal O2•− production in renal homogenates that is dependent on the stimulation of the AT1 R and NADPH oxidase. Moreover, the stimulation of AT1 R increases the NADPH oxidase–dependent superoxide generation, and this effect may contribute to the renal excretory and hemodynamic actions of Ang II.

In summary, the data in the present study indicate that there is within the kidney a basal NADPH oxidase–dependent generation of O2•− that reduces NO bioavailability and modulates renal function. In addition, this NADPH oxidase–dependent generation of O2•− is stimulated by Ang II and contributes to the acute renal actions of this peptide.

Perspectives
In the past few years, increasing evidence indicates that oxygen-derived free radicals modulate vascular function and arterial pressure in several models of arterial hypertension. Because the kidney and specifically the pressure-natriuresis phenomenon is thought to play a central role in the long-term control of arterial pressure, a more precise understanding of the renal mechanisms involved in the effects of free radicals influencing blood pressure regulation will be necessary to improve our knowledge of the pathophysiology of arterial hypertension.

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