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Superoxide mediates acute renal vasoconstriction produced by angiotensin II and catecholamines by a mechanism independent of nitric oxide

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Just A, Olson AJ, Whitten CL, Arendshorst WJ. Superoxide mediates acute renal vasoconstriction produced by angiotensin II and catecholamines by a mechanism independent of nitric oxide. *Am J Physiol Heart Circ Physiol* 292: H83–H92, 2007. First published September 1, 2006; doi:10.1152/ajpheart.00715.2006.—NAD(P)H oxidases (NOX) and reactive oxygen species (ROS) are involved in vasoconstriction and vascular remodeling during hypertension produced by chronic angiotensin II (ANG II) infusion. These effects are thought to be mediated largely through superoxide anion (O_2^-) scavenging of nitric oxide (NO). Little is known about the role of ROS in acute vasoconstrictor responses to agonists. We investigated renal blood flow (RBF) reactivity to ANG II (4 ng), norepinephrine (NE, 20 ng), and α -adrenergic agonist phenylephrine (PE, 200 ng) injected into the renal artery (ira) of anesthetized Sprague-Dawley rats. The NOX inhibitor apocynin ($1-4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ira, 2 min) or the superoxide dismutase mimetic Tempol ($1.5-5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ira, 2 min) rapidly increased resting RBF by $8 \pm 1\%$ ($P < 0.001$) or $3 \pm 1\%$ ($P < 0.05$), respectively. During NO synthase (NOS) inhibition (*N*^ω-nitro-L-arginine methyl ester, 25 mg/kg iv), the vasodilation tended to increase (apocynin $13 \pm 4\%$, Tempol $10 \pm 1\%$). During control conditions, both ANG II and NE reduced RBF by $24 \pm 4\%$. Apocynin dose dependently reduced the constriction by up to 44% ($P < 0.05$). Similarly, Tempol blocked the acute actions of ANG II and NE by up to 48–49% ($P < 0.05$). In other animals, apocynin ($4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ira) attenuated vasoconstriction to ANG II, NE, and PE by 46–62% ($P < 0.01$). During NOS inhibition, apocynin reduced the reactivity to ANG II and NE by 60–72% ($P < 0.01$), and Tempol reduced it by 58–66% ($P < 0.001$). We conclude that NOX-derived ROS substantially contribute to basal RBF as well as to signaling of acute renal vasoconstrictor responses to ANG II, NE, and PE in normal rats. These effects are due to O_2^- rather than H_2O_2 , occur rapidly, and are independent of scavenging of NO.

hemodynamics; vascular smooth muscle; renal vascular resistance; afferent arteriole; oxidative stress; reactive oxygen species; redox signaling

REACTIVE OXYGEN SPECIES (ROS) and superoxide anion (O_2^-) generated by NAD(P)H oxidase (NOX) contribute importantly to the regulation of vascular tone, particularly in pathophysiological states such as hypertension and ischemic tissue injury (4, 14, 23, 40, 53). Endothelial dysfunction, characterized by increased vascular expression of NOX subunits and production of O_2^- and impaired endothelial vasomotor function largely due to inactivation of the vasodilator nitric oxide (NO) by O_2^- , is a

hallmark sign and predictive of cardiovascular risk and end-organ damage (14, 53, 56). ROS are thought to be involved in the pathogenesis and maintenance of hypertension by affecting renal function and promoting salt and fluid retention (4, 23, 35). The specific mechanisms are not clear, although evidence indicates that ROS can impair endothelium-dependent vasodilation and favor renal vasoconstriction (37, 42, 54) and increase sodium reabsorption, at least in the thick ascending limb of Henle's loop (25, 33). Two-week exposure to angiotensin II (ANG II) upregulates NOX-1 and p22^{phox} mRNA in vascular smooth muscle cells (VSMC) and the renal cortex (3, 4).¹

Low physiological levels of ROS are thought to be involved in cell signaling (8, 11). However, the involvement of ROS in regulation of renal hemodynamics during basal resting conditions and acute renal vascular responses to ANG II and other vasoconstrictors in healthy animals is less well characterized than in chronic disease conditions. Initial reports have been conflicting; some give the impression that acute physiological effects may be less NO dependent from the common endothelial dysfunction associated with disease. Although not a universal finding, several studies find that NOX-dependent ROS contribute to the regulation of resting vasomotor tone in the kidney under basal conditions (5, 13, 27, 29, 30, 59, 60). Less clear is the involvement of NOX and ROS in acute vasoconstrictor responses. Support is provided by evidence that NOX activity and O_2^- production mediate at least some acute ANG II, endothelin-1 (ET-1) and thromboxane A_2 signaling in healthy renal resistance vessels in vivo and in vitro (9, 10, 31, 34, 42, 59). On the other hand, other investigators report that either apocynin or Tempol is ineffective in modulating resting renal vasomotor tone in normal animals (7, 15, 27, 30, 31); nevertheless, they attenuate acute renal vasoconstriction induced by ANG II infusion (7, 27) and responses of isolated vascular preparations to multiple agonists (54).

In vivo and in vitro studies indicate that O_2^- and NO interact in the regulation of afferent arteriolar tone by tubuloglomerular feedback (TGF) in normal rats and rabbits (26, 36, 55, 56). In this regard, NO generated by neuronal NO synthase (nNOS) expressed in macula densa (MD) cells blunts the magnitude of

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the TGF response at rapid fluid flow rates. On the other hand, O_2^- formed in the juxtaglomerular apparatus limits NO signaling, an action that is reduced acutely by Tempol (36, 56). This SOD mimetic is reported to be ineffective during inhibition of nNOS, suggesting that the major action of O_2^- is to neutralize the vasodilator NO, perhaps within MD cells (36, 56). Addition of catalase has no additional effect to that of Tempol alone, suggesting mediation by O_2^- rather than H_2O_2 (26). Other evidence, however, supports the view that that O_2^- may potentiate TGF by exerting a constrictor-like action directly on the afferent arteriole independent of local NO (26). The role of these actions and interactions in integrated responses at the whole kidney level requires further investigation.

The present renal blood flow (RBF) study was undertaken to gain insight into the importance of NOX activity and O_2^- production in VSMC reactivity and cellular signaling of resistance arterioles of healthy kidneys of normotensive Sprague-Dawley rats. Renal vascular tone and responsiveness were determined under basal conditions and during acute stimulation with ANG II, norepinephrine (NE), or phenylephrine (PE) injected into the renal artery. We tested the hypotheses that these vasoconstrictor agents rapidly stimulate NOX activity to produce O_2^- that in turn mediates a significant portion of the immediate renal vasomotor response. We also assessed whether the acute renal hemodynamic effects of O_2^- are dependent on the presence of NO or persist during inhibition of NO production. The effects of O_2^- were distinguished from those of H_2O_2 by using apocynin to inhibit NOX subunit assembly and Tempol as a SOD mimetic.

METHODS

Experiments conducted on 37 male Sprague-Dawley rats (6–8 wk of age, 170–280 g body wt) from our local breeding colony were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. The study was performed according to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and the guidelines of the Animal Welfare Act. The animals were fed a standard lab chow with free access to tap water and kept on a 12-h:12-h light-dark cycle. Surgical preparation and general methods were similar to those described previously (16).

Surgical Preparation

After induction of anesthesia by pentobarbital sodium (Nembutal, 50–60 mg/kg body wt ip; Abbott, Chicago, IL), a rat was placed on a temperature-controlled table kept at 37°C. The depth of anesthesia was monitored by the response to ear or toe pinching. The left femoral artery was catheterized for measurement of arterial pressure (AP), and two femoral venous catheters were used for infusion of volume replacement and injection of pentobarbital. The trachea was cannulated to facilitate respiration. Via a midline abdominal incision, the aorta and left renal artery were exposed. A catheter was inserted into the left common iliac artery and advanced until its tip faced the origin of the left renal artery and used for infusion into the renal artery. An ultrasound transit-time flow probe (model 1RB, Transonic, Ithaca, NY) was placed around the left renal artery and filled with ultrasonic coupling gel (Surgilube, Fugera, Melville, NY). Urine was drained from the bladder by gravity via a 23-gauge needle. Isoncotic bovine serum albumin (4.75 g/dl) was infused initially at 50 μ l/min to replace surgical losses (1.25 ml/100 g body wt), followed by a maintenance rate of 10 μ l/min. The renal artery catheter was perfused with isotonic saline at 5 μ l/min. Additional doses of pentobarbital were given intravenously as required. All syringes and catheters used for solu-

tions injected/infused into the renal artery (ira) were pretreated with albumin solution (0.5 g/dl) to reduce surface adhesion. At least 60 min were allowed after surgery before starting an experiment.

Measurements

Femoral AP was measured via a pressure transducer (model P23 DB, Statham). RBF was measured by a flowmeter (model T-420, Transonic, low-pass filter 40 Hz). Zero offset was determined at the end of an experiment after cardiac arrest. AP and RBF were recorded on a computer (Pentium IV + DataTranslation A/D converter + Labtech Notebook-Pro 12.1) at 100 Hz and stored at 1 Hz as consecutive mean values over 1-s periods. AP was also stored at 100 Hz for determination of heart rate.

Protocols

The RBF response to a bolus injection of ANG II (10 μ l \times 0.38 μ M = 3.8 pmol or 4 ng), norepinephrine (NE, 10 μ l \times 9.7 μ M = 97 pmol or 20 ng), or phenylephrine (PE, 10 μ l \times 98 μ M = 1 nmol or 200 ng) into the renal artery was measured during control and experimental conditions. Agonist doses were chosen to induce a reduction of RBF by 20–40%. Two minutes before each bolus injection, the renal arterial infusion rate was increased from 5 to 140 μ l/min. A 10- μ l bolus of ANG II or NE was then injected into the infusion line through a microinjector valve (Valco Instruments, Houston, TX), and a new recording was started. The initial 25 s served as baseline values of AP and RBF. At 150 s after the bolus, the infusion rate was returned to 5 μ l/min, and the recording continued for another 4 min. At least 5 min were allowed for recovery after each injection.

We used apocynin (acetovanillone, 4'-hydroxy-3'-methoxy-acetophenone) to block NOX activity and Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) as a cell-permeable SOD mimetic to convert O_2^- to H_2O_2 . Apocynin is known to block NOX subunit assembly and thus activity (49). Apocynin markedly reduces O_2^- production in the media of the aortic wall (6, 32), with complete inhibition noted in cultured aortic VSMC or cultured gluteal arterial VSMC stimulated by ANG II (52, 58). We observe that apocynin blocks >90% of O_2^- produced (tempo-9AC fluorescence) in isolated afferent arteriolar segments in response to stimulation by ANG II and ET-1 (9, 10). Tempol reduces intracellular O_2^- concentration >90% in the same preparation.

Dose-response relations were established in our current RBF studies with the target of producing significant intrarenal inhibition in the absence of changes in systemic AP. The highest dose of apocynin infusion in our studies was ~50% of that used in a previous rat study (27). The highest Tempol infusion rate was 10 times greater than that reported (0.5 mg \cdot kg⁻¹ \cdot min⁻¹ ira) to markedly reduce urinary excretion of 8-isoprostane in dogs (30) and two times greater than that previously infused into the renal artery of rats (27).

Experimental Groups

Apocynin ($n = 6$). We tested the effect of reducing the levels of both O_2^- and H_2O_2 on reactivity to ANG II and NE. RBF responses were tested in the absence and presence of different doses of the NOX inhibitor apocynin during control (vehicle, 8% ethanol ira), apocynin (ira infusion at 1, 2, and 4 mg \cdot kg⁻¹ \cdot min⁻¹ in 8% ethanol in ascending or descending order), and recovery (vehicle ira) periods.

Tempol ($n = 6$). To study the involvement of ROS by scavenging of O_2^- , renal reactivity was determined in the absence and presence of different doses of the SOD mimetic Tempol in saline. This is expected to reduce the level of O_2^- while possibly raising that of H_2O_2 . Reactivity to ANG II and NE was tested during control (saline ira), Tempol (ira infusion at 1.5, 3, and 5 mg \cdot kg⁻¹ \cdot min⁻¹ in ascending or descending order), and recovery (saline) periods. In two rats, tempo (2,2,6,6-tetramethylpiperidine 1-oxyl) was used in 1.5% DMSO instead of Tempol. As responses were similar, the results were pooled.

Apocynin-B ($n = y$). Responses to ANG II, NE, and PE were tested during control (8% ethanol ira), apocynin high dose (4 mg·kg⁻¹·min⁻¹ ira in 8% ethanol), and recovery (ethanol ira) periods.

Apocynin during NOS inhibition [N^ω-nitro-L-arginine methyl ester (L-NAME) + apocynin; n = 8]. To assess the dependence of the effect of apocynin on the presence of NO, reactivity to ANG II and NE was evaluated during the following periods: control (8% ethanol ira), control II (vehicle ira) 15 min after injection of the NOS inhibitor L-NAME (25 mg/kg in 1 ml/kg iv saline), apocynin (4 mg·kg⁻¹·min⁻¹ in ethanol) 30 min after L-NAME, and recovery (vehicle ira). This very high dose of L-NAME was selected because it is known to produce near maximum inhibition of NOS as evidenced by a maximum increase in mean AP and reduction in RBF (1, 16).

Tempol during NOS inhibition (L-NAME + Tempol, n = 7). To study the importance of NO for the effect of Tempol, renal vascular reactivity to ANG II and NE was tested during the following periods: control (saline ira), control II (saline ira) 15 min after L-NAME (25 mg/kg iv), Tempol (5 mg·kg⁻¹·min⁻¹ ira) 30 min after L-NAME, and recovery (saline ira).

The time course of apocynin and Tempol effects was calculated as the difference between RBF responses in control and experimental periods divided by the control RBF reduction to ANG II and NE.

Drugs and Chemicals

ANG II, PE, Tempol, tempo, apocynin, L-NAME, and albumin were obtained from Sigma (St. Louis, MO). NE (Levophed) was from Abbott Laboratories (Chicago, IL).

Data Analyses

The maximum RBF decrease following each injection was determined off-line by custom-built software from the 1-Hz data after smoothing by sliding average over five values. The change was expressed as percentage of the baseline value. Baseline RBF and AP were determined from the average of the first 25 s of each recording immediately before injection. To obtain mean time courses, the original 1-Hz recordings (without smoothing) were averaged for each experimental period of all animals in a group. HR was determined from the 100-Hz recording of AP off-line. Data are expressed as means ± SE. Statistical significance among groups was tested by ANOVA in conjunction with Holm-Sidak or Tukey test for multiple comparisons (SigmaStat 3.00, SPSS, Chicago, IL). In case of nonnormal distribution, data were transformed by square root before analysis. Paired *t*-test was used to detect changes within a group. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline hemodynamic data at the beginning of the experiment and urine excretion rate during the observation period are presented in Table 1.

To determine whether O₂⁻ influenced basal RBF, apocynin was infused into the renal artery for 2 min to inhibit NOX activity; in other animals, the SOD mimetic Tempol was also infused into the renal artery. Each caused an immediate increase in RBF (Fig. 1). Apocynin (4 mg·kg⁻¹·min⁻¹) (pooled from apocynin and apocynin-B groups) caused rapid vasodilation, increasing RBF by 8 ± 1% ($P < 0.001$ vs. 0 and vs. its vehicle) over the 2-min observation period. Scavenging of O₂⁻ by Tempol (5 mg·kg⁻¹·min⁻¹) increased RBF by 3 ± 1% ($P < 0.05$ vs. zero). The time course was almost identical to that of apocynin, although the magnitude of the dilation was smaller ($P < 0.05$). The vehicle used for apocynin (8% ethanol) tended to reduce RBF (-2 ± 1%, $P > 0.07$ vs. 0).

In two other groups, the influence of O₂⁻ on basal renal hemodynamics was tested during inhibition of NO production using L-NAME. During NOS inhibition, apocynin increased RBF by 13 ± 4% ($P < 0.001$ vs. vehicle, $P < 0.01$ vs. 0), and Tempol increased RBF by 10 ± 1% ($P < 0.01$ vs. 0) (Fig. 1). Both effects tended to be almost twice as large as in the presence of NO. The difference was greater for Tempol ($P < 0.05$), but that for apocynin was more variable and did not attain statistical significance.

To study the involvement of O₂⁻ and H₂O₂ in the acute constrictor responses to ANG II and to sympathoadrenergic stimulation, ANG II (4 ng in 10 μl), NE (20 ng), and PE (200 ng) were injected into the renal artery before and during intrarenal infusion of apocynin or Tempol.

Increasing doses of apocynin (1–4 mg·kg⁻¹·min⁻¹ ira) diminished the renal vasoconstriction produced by ANG II in a dose-dependent manner, from a maximum reduction of RBF of 26 ± 3% to 15 ± 3% ($P < 0.01$, Fig. 2, *top left*). Apocynin had a similar inhibitory effect on the constrictor response to NE. The control response of 23 ± 3% was reduced to 12 ± 1% ($P < 0.01$, Fig. 2, *top right*). Likewise, similar results were found when the SOD mimetic Tempol was used (1.5–5 mg·kg⁻¹·min⁻¹ ira) to scavenge O₂⁻. The highest dose of Tempol reduced renal vasoconstriction elicited by ANG II, from 24 ± 4% to 12 ± 1% ($P < 0.01$, Fig. 2, *bottom left*), and that by NE from 24 ± 4% to 13 ± 4% ($P < 0.05$, Fig. 2, *bottom right*). The inhibitory actions of apocynin and Tempol were rapidly reversible. The responsiveness to ANG II and NE was restored to basal levels during a recovery period (Fig. 2).

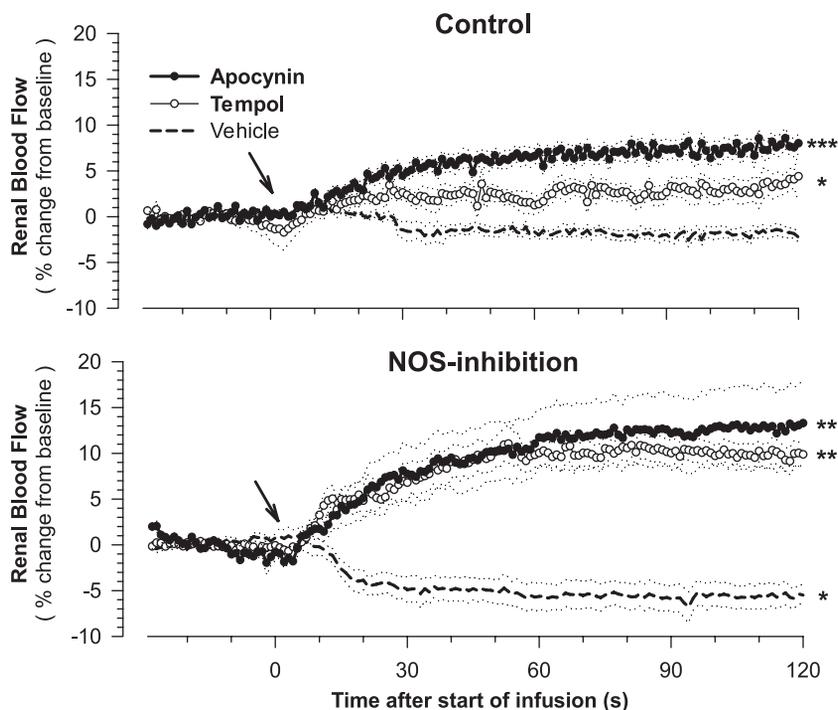
Because NE stimulates both α- and β-adrenergic receptors, we also tested the influence of O₂⁻ on acute reactivity to specific α₁-adrenergic stimulation by PE. In this series, renal vasoconstriction elicited by PE was attenuated by apocynin (4 mg·kg⁻¹·min⁻¹) from 25 ± 5% to 9 ± 1% ($P < 0.01$, Fig. 3, *right*). In the same animals, apocynin reduced the constrictor

Table 1. Baseline hemodynamic and excretory data

Experimental Group	n	MAP, mmHg	RBF, ml·min ⁻¹ ·g ⁻¹	HR, beats/min	Hct, %	V, μl/min
Apocynin	6	102±4	6.8±0.6	312±22	42±1	21±3
Tempol	6	110±4	5.3±0.3	283±13	41±1	37±6
Apocynin-B	10	90±3	4.2±0.3	280±9	43±1	26±4
L-NAME + apocynin	8	100±3	4.5±0.3	287±11	44±1	34±5
L-NAME + Tempol	7	109±3	5.0±0.3	295±6	41±1	37±3

Values are means ± SE for *n* rats. MAP, mean arterial pressure; RBF, renal blood flow; HR, heart rate; Hct, hematocrit, during the control period; V, bilateral urine flow rate averaged over the entire experiment. For descriptions of experimental groups, see METHODS.

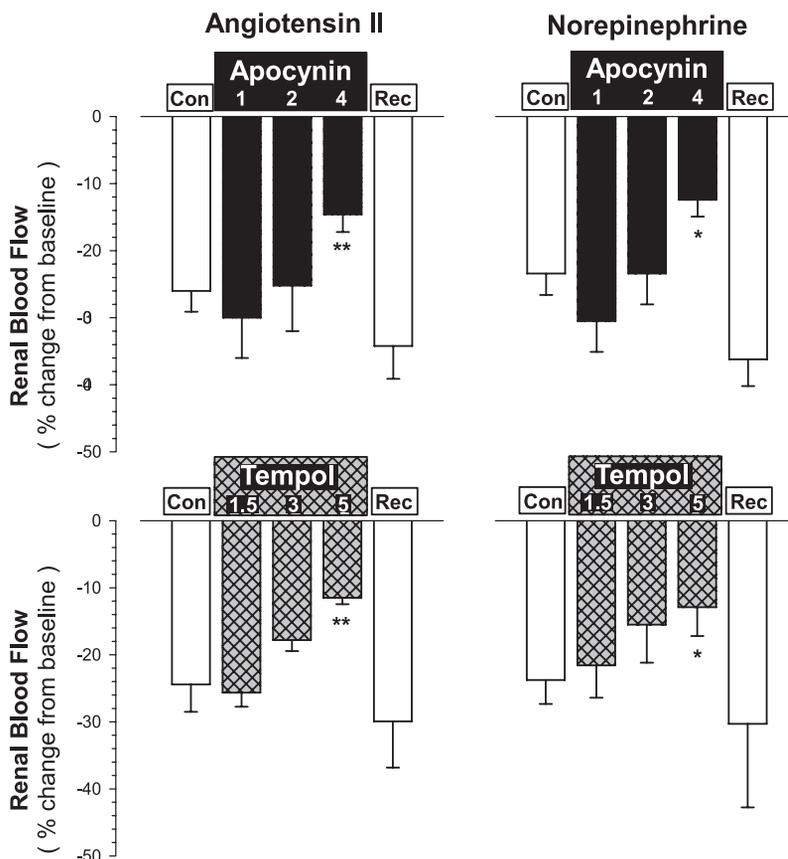
Fig. 1. Time course of the vasodilatory effect of apocynin and Tempol on baseline renal blood flow (RBF) of anesthetized rats during control and during nitric oxide (NO) synthase (NOS) inhibition. *Top*: the NAD(P)H-oxidase inhibitor apocynin ($4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $n = 16$, solid circles), the superoxide dismutase mimetic Tempol ($5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $n = 6$, open circles), or the vehicle for apocynin (8% ethanol, $n = 16$, broken line) was infused separately into the renal artery during control conditions, starting at time (t) = 0 s. Vehicle for Tempol was saline, which did not change RBF and is not shown. *Bottom*: apocynin ($n = 8$), Tempol ($n = 7$), and vehicle ($n = 8$) were infused separately during NOS inhibition [N^{ω} -nitro-L-arginine methyl ester (L-NAME), 25 mg/kg iv]. Stippled lines indicate \pm SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0.



response to ANG II from $32 \pm 4\%$ to $16 \pm 2\%$ ($P < 0.05$, Fig. 3, *left*) and that to NE from $39 \pm 5\%$ to $14 \pm 2\%$ ($P < 0.05$, Fig. 3, *middle*), confirming results of earlier experiments. Because apocynin elevated baseline RBF, we also analyzed absolute changes in RBF. Apocynin attenuated ANG II-in-

duced reduction of RBF from 1.3 ± 0.2 to $0.7 \pm 0.1 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($P < 0.05$), and that to NE from 1.6 ± 0.3 to $0.6 \pm 0.1 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($P < 0.01$). The PE response was reduced from 1.1 ± 0.2 to $0.5 \pm 0.1 \text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($P < 0.05$).

Fig. 2. Apocynin and Tempol exhibit dose-dependent inhibition of renal vasoconstrictor responses to angiotensin II (ANG II) and norepinephrine (NE). Maximum reduction of RBF in response to intrarenal arterial (ira) injection of ANG II (4 ng ira, *left*) or NE (20 ng ira, *right*) before [control (Con), open bars], during escalating doses of apocynin (solid bars, *top*), or Tempol (crosshatched bars, *bottom*), followed by a recovery period (Rec; open bar). Apocynin was infused at 1, 2, and $4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ira ($n = 6$). Tempol was infused at 1.5, 3, and $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ira ($n = 6$). Values are means \pm SE. * $P < 0.05$, ** $P < 0.01$ vs. initial control.



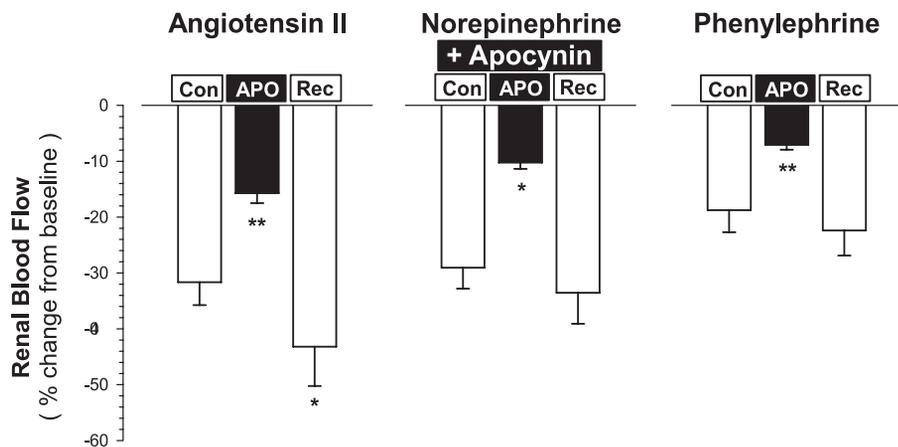


Fig. 3. Effect of apocynin (Apo) on the renal vasoconstrictor responses to ANG II, NE, and phenylephrine (PE). Maximum reduction of RBF in response to injection of ANG II (4 ng ira), NE (20 ng ira), and PE (200 ng ira) before (Con, open bar), during apocynin (4 mg·kg⁻¹·min⁻¹, solid bar), and in a subsequent recovery period (Rec, open bar). Values are means ± SE; n = 10. *P < 0.05, **P < 0.01 vs. initial control.

To determine whether the actions of O₂⁻ are secondary to scavenging of NO, we tested the vasoconstrictor responses to ANG II and NE during apocynin or Tempol treatment combined with inhibition of NOS. For this purpose, we used a very high dose of L-NAME (25 mg/kg iv) that produces near complete inhibition of NOS production as evidenced by maximum changes in mean AP and RBF (1, 16). Inhibition of NOS reduced baseline RBF by 34 ± 6% (P < 0.01) and increased mean AP by 50 ± 3 mmHg (P < 0.001). The renal vasoconstrictor response to ANG II was magnified (54 ± 6 vs. 28 ± 4%, P < 0.05) as was that to NE (86 ± 6 vs. 48 ± 6%, P < 0.001). In the absence of NO, apocynin (4 mg·kg⁻¹·min⁻¹ ira)

increased baseline RBF by 13% (Fig. 1, P < 0.05 vs. 0). This NOX inhibitor markedly attenuated the constrictor responses to both ANG II (17 ± 2 vs. 54 ± 6%, P < 0.01) and NE (23 ± 2 vs. 86 ± 6%, P < 0.001, Fig. 4) during L-NAME inhibition of NOS. Similar results were noted for absolute changes in RBF. The RBF response to ANG II during L-NAME was reduced by apocynin from 1.8 ± 0.4 to 0.6 ± 0.1 ml·min⁻¹·g⁻¹ (P < 0.05), and that to NE from 2.7 ± 0.3 to 0.8 ± 0.1 ml·min⁻¹·g⁻¹ (P < 0.001).

Analogous results were observed when Tempol (5 mg·kg⁻¹·min⁻¹ ira) was used to scavenge O₂⁻ during NOS inhibition. In this group, L-NAME alone reduced basal RBF by

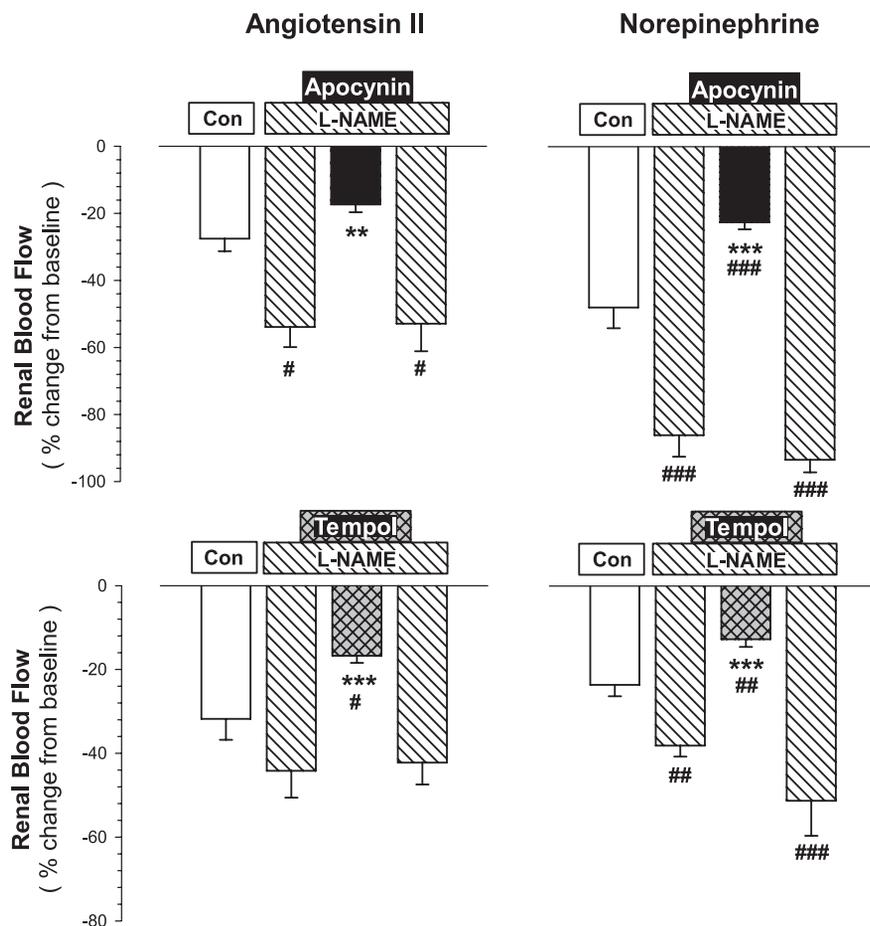
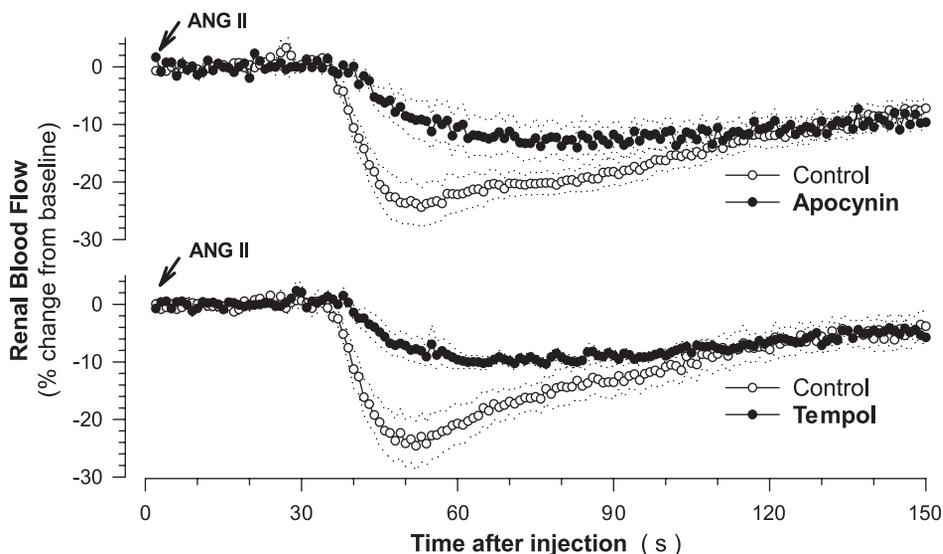


Fig. 4. Inhibitory effect of apocynin and Tempol on acute renal vasoconstriction produced by ANG II and NE in the presence and absence of NO. RBF response to intrarenal injection of ANG II (4 ng, left) or NE (20 ng, right) before (Con, open bar), during NOS inhibition alone (L-NAME, 25 mg/kg iv, gray crosshatched bar), during additional infusion of apocynin (4 mg·kg⁻¹·min⁻¹ ira; n = 8; top, solid bar), or Tempol (5 mg·kg⁻¹·min⁻¹ ira, n = 7, bottom, crosshatched bar), and in a subsequent recovery period during NOS inhibition alone (Rec, matched bar). Values are means ± SE. **P < 0.01, ***P < 0.001 vs. initial L-NAME alone; #P < 0.05, ###P < 0.01, ####P < 0.001 vs. initial control.

Fig. 5. Time course of the renal vasoconstrictor response to ANG II before and during apocynin and before and during Tempol. *Top*: RBF responses to bolus injection of ANG II (4 ng) into the renal artery during concomitant infusion of vehicle (control, open circles) or apocynin (4 mg·kg⁻¹·min⁻¹ i.v., solid circles; *n* = 6). *Bottom*: RBF response to ANG II before (control, open circles) and during intra-renal arterial infusion of Tempol (5 mg·kg⁻¹·min⁻¹, solid circles, *n* = 6). ANG II was injected at *t* = 0. The initial delay of ~30 s is due to travel time through the catheter before reaching the renal vasculature. Stippled lines denote ± SE.



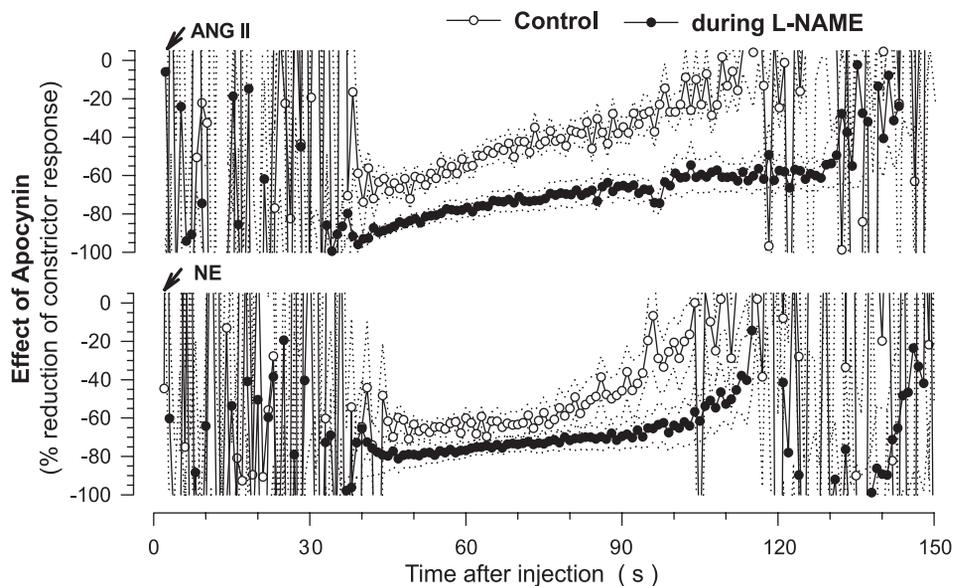
44 ± 2% (*P* < 0.001), increased mean AP by 40 ± 5 mmHg (*P* < 0.05), and augmented NE-induced renal vasoconstriction (38 ± 3 vs. 24 ± 4%, *P* < 0.01, Fig. 4, *right*) as it tended to do for ANG II (44 ± 6 vs. 32 ± 5%, *P* > 0.09, Fig. 4, *left*). During continued NOS inhibition, Tempol elevated basal RBF by 10% (*P* < 0.05 vs. 0; Fig. 1) and strongly reduced the responsiveness to both ANG II (17 ± 2 vs. 44 ± 6%, *P* < 0.001; Fig. 4, *bottom left*) and NE (13 ± 2 vs. 23 ± 3%, *P* < 0.001; Fig. 4, *bottom right*). The same was true for absolute changes in RBF because the constrictor response to ANG II was attenuated by Tempol from 1.2 ± 0.2 to 0.5 ± 0.1 ml·min⁻¹·g⁻¹ (*P* < 0.05), and that to NE from 1.0 ± 0.1 to 0.4 ± 0.1 (*P* < 0.001).

Typical time courses of the renal vasoconstriction produced by ANG II are shown in Fig. 5. ANG II caused a transient reduction of RBF, reaching its maximum of 20–30% below baseline ~15 s after the first response is apparent. The initial lag time of 30 s is due to the transit of the bolus through the renal arterial catheter. In the present

study, the maximum RBF responses were compared among treatment groups. By design, AP was stable during each injection, and thus RBF changes reflect reciprocal changes in renal vascular resistance.

The time course of the inhibitory effect of apocynin, taken as the difference between control and experimental RBF curves relative to the control response, is shown in Fig. 6. Similar kinetics and degrees of inhibition are seen for ANG II and NE. Inhibition of ~50% is clearly evident very rapidly, almost immediately once agonist-induced vasoconstriction occurs. An O₂⁻ effect is maintained throughout the constrictor phase (35–60 s) and tends to wane when RBF returns toward control levels (60–120 s). The constrictor influence of O₂⁻ is even more prominent during inhibition of NOS, increasing early during constriction from ~50 to 85%. The effect tends to persist longer into the recovery phase, compatible with an ability of NO to scavenge O₂⁻ after maximum constriction is achieved when NOS is not inhibited.

Fig. 6. Time course of the apocynin effect during renal vasoconstriction produced by ANG II and NE before and during inhibition of NOS using L-NAME. *Top*: constrictor influence of O₂⁻ revealed by apocynin effect during ANG II-induced renal vasoconstriction. *Bottom*: influence of O₂⁻ evidenced by apocynin effect during NE-induced renal vasoconstriction. Apocynin effect is calculated as the difference between agonist-induced change in RBF during control and experimental periods normalized to the change in RBF during control. Values are means ± SE. Data were pooled from apocynin and apocynin-B groups (*n* = 16).



DISCUSSION

The aims of the present study were to determine whether ROS, in particular, O_2^- , exert a tonic influence on basal renal hemodynamics at rest and to evaluate the extent to which O_2^- contributes to acute renal vasoconstriction produced by ANG II, NE, and PE in normal Sprague-Dawley rats. Our findings demonstrate that constitutive production of endogenous ROS in the vasculature contributes to the baseline level of blood flow in the kidney of euvoletic rats. Furthermore, ROS participate importantly in the renal vasoconstrictor response to acute stimulation with ANG II that reduces RBF ~30% of baseline. ROS are also involved in the acute renal vasomotor effects of the α -adrenoceptor agonists NE and PE. These influences of ROS, both on baseline RBF as well as on vasoconstriction elicited by agonists, are mediated by O_2^- rather than H_2O_2 , as demonstrated by the similarity of results with apocynin and Tempol. In addition, our results show that neither of these actions of O_2^- is dependent on the availability of NO and thus cannot be mediated by scavenging of superoxide by NO or vice versa. Accordingly, both actions reflect direct constrictor effects of O_2^- on the renal resistance arterioles during basal conditions and acute stimulation by ANG II, NE, and PE. This action of O_2^- and its buffering by either NOX inhibition or the SOD mimetic are very rapid, apparent as soon as constriction by ANG II and NE is detected and persisting during the constrictor phase whether or not NOS was inhibited. It is important to appreciate that all agents were administered into the renal artery in the absence of changes in AP for the purpose of assessing local intrarenal effects in the absence of potentially complicating systemic influences.

Tonic Renal Vasoconstriction Mediated By O_2^-

Vascular O_2^- production is primarily NOX dependent, and NOX systems expressed in VSMC exhibit basal oxidase activity supported by both NADH and NADPH (6, 22). NOX-4 appears to be constitutively active in cultured VSMC. Homogenates of renal cortex and outer medulla produce significant amounts of O_2^- via NOX under basal, unstimulated conditions (27, 60).

Our RBF results show that acute inhibition of O_2^- production by the NOX inhibitor apocynin, as well as scavenging of O_2^- by the stable membrane-permeable SOD mimetic Tempol, induces rapid and significant decreases in baseline renal vascular resistance. Intrarenal infusion of apocynin produced a modest but consistent ~8% vasodilator effect within the 2-min observation period, whereas Tempol increased RBF by 3% on the average. Because both NOX inhibition and O_2^- scavenging increase RBF, it is likely that the results are primarily due to O_2^- rather than H_2O_2 . It is noteworthy that apparent maximum inhibition was achieved in our experiments within 60–90 s. By design, we chose doses of intrarenally infused agents that did not affect systemic AP and, therefore, may have exerted less than maximal local renal effects. Differences in degree of enzyme inhibition may also explain why Tempol had less of an effect on baseline RBF compared with that of apocynin in our studies. Alternatively, apocynin blocks production of O_2^- , whereas SOD reduces O_2^- and increases H_2O_2 along with downstream metabolites that may be vasoactive. To our knowledge, the initial time courses of the acute integrated renal vascular actions of apocynin and Tempol have not been re-

ported previously. We infused these agents into the renal artery during continuous measurement of RBF. The observed kinetics over 60–90 s reflect the net effects of drug uptake and putative metabolic effects, such as inhibition of endogenous O_2^- production, whereas degradation continues in the case of apocynin and increased SOD activity during administration of Tempol. Clearly, this is a dynamic system capable of responding rapidly to stimuli. A previous assessment of local Tempol effects in the renal medulla over time indicates that this SOD mimetic increases blood flow within the initial 20 min, with maximal effects stable between 20 and 60 min (60).

To our knowledge, only one previous animal study has tested the acute effects of NOX inhibition on basal renal vasomotor tone. This report showed ~30% increase in RBF during 10-min infusion of a NOX inhibitor into the renal artery of anesthetized Sprague-Dawley rats (27). The dose of apocynin used in that particular study was twice that which we employed and tended to reduce systemic AP unless mesenteric and celiac arteries were occluded. Previous studies have reported that intrarenal infusion of Tempol increases or inhibition of SOD reduces blood flow in the kidney, suggesting a basal tonic level of ROS production (5, 13, 29). Nevertheless, others have noted minimal acute effects of intrarenal infusion of Tempol on basal RBF in anesthetized dogs and rats (19, 20, 27, 30, 31). Neither acute nor chronic (1–2 wk) systemic administration of Tempol has an influence on basal RBF or AP in normotensive rats or mice (7, 17, 41, 43). The reasons for the variability in results among studies are not known. It is recognized that steady-state responses to systemic administration may reflect a combination of systemic as well as renal effects. We think it is likely that the relatively small but highly reproducible changes ($\leq 10\%$) we observed are statistically significant because our systematic studies were paired in the same animals and because immediate responses were detected by continuous RBF recordings made by a very sensitive system compared with unpaired observations between groups of animals.

In the rat renal medulla, Tempol increases basal blood flow by ~33%, and inhibition of SOD with diethyldithiocarbamate (DETC) decreases blood flow by a similar amount. Both acute responses are consistent with a strong vasoactive effect of O_2^- during basal conditions (60). In another study, Tempol-induced renal vasodilation was more readily apparent during catalase treatment (5).

In vitro studies of renal arteries and arterioles of rabbits and rats have generally failed to unmask an acute effect of Tempol on basal vasomotor tone. This holds for the afferent arteriole of the juxtamedullary nephron preparation, isolated-perfused rat chronically hydronephrotic kidney, and isolated-perfused rabbit arterioles (15, 34, 54).

O_2^- -Mediated ANG II-Induced Renal Vasoconstriction

We found that the overall intrarenal vascular action of O_2^- during agonist stimulation and its buffering by either NOX inhibition or the SOD mimetic are very rapid, essentially discernable when constriction by ANG II and NE is first detected, with pronounced effects immediately, persisting during the constrictor phase, and waning during recovery of RBF toward baseline. During NOS inhibition, the contribution of O_2^- was similar or greater, based on the apocynin

effect, to ANG II- and NE-induced renal vasoconstriction. The constrictor influence due to O_2^- started immediately and was maintained beyond the maximum decrease in RBF in the absence of NO.

The present study establishes that NOX-derived O_2^- is importantly involved in the G protein-coupled receptor signaling pathways resulting in acute vasoconstriction in the kidney. This is demonstrated by the attenuation of the ANG II-, NE-, and PE-mediated renal vasoconstriction by at least 50% by either apocynin or Tempol infused into the renal artery. These receptor agonists appear to very rapidly stimulate O_2^- production that mediates constriction, as attenuation by apocynin or Tempol is evident as soon as the agonist reaches resistance arterioles and vasoconstriction is observed (Figs. 2 and 3). The similarity of the degree of inhibition of agonist-induced constriction with apocynin and Tempol indicates that the involvement of ROS is predominantly due to O_2^- rather than H_2O_2 . To the extent that the highest doses employed in the present study produce less than maximum inhibition, our results underestimate the intrarenal influence of O_2^- .

Recent evidence from other laboratories supports the notion that NOX activity and O_2^- production mediate some of the ANG II signaling in healthy renal resistance vessels, although results to date are mixed on this point. As we observed in the rat, the free radical scavenger Tempol is reported to block ~50% of acute renal vasoconstriction produced by intrarenal infusion of ANG II in the dog (31). Other rat studies did not find a discernable Tempol effect on constriction on the renal circulation produced by ANG II infused into the renal artery (7, 27). Also, although apocynin was administered at a high dose that increased basal RBF, the magnitude of renal vasoconstriction produced by ANG II infusion was unaffected (27). The reasons for these differences in results are not clear and warrant further investigation. As mentioned earlier, our paired design using each animal as its own control might have contributed to smaller variation of acute responses in our study.

Vascular NOXs also have agonist-stimulated activity (6, 48). NOX activity can be stimulated by agonists acutely (in s-min) and also have subunit expression upregulated chronically (in h-days) to produce ROS, by apparently different mechanisms. ANG II is known to rapidly increase O_2^- generation in aortic segments (18, 24, 46, 47), isolated VSMC (45), and renal cortical homogenates (27). Inhibition of PKC profoundly inhibits the early activation phase of NOX in response to ANG II, whereas the later sustained phase is only partially inhibited (45). Acutely, NOX-1 located in caveolae in VSMC and NOX-2 in endothelial cells are activated by PKC-, PLA₂-, and cSrc-dependent translocation of p47^{phox} to the plasma membrane. Use of sensitive fluorescent dyes reveals that ANG II can rapidly stimulate AT₁ receptors and PKC to phosphorylate and translocate p47^{phox} to the plasma membrane. As a result, NOX is activated to generate intracellular O_2^- and H_2O_2 in VSMC, effects inhibited DPI or AT₁ receptor antagonist, SOD, and the SOD analog tiron (6, 38, 39, 45, 52) (18). We have recently shown this to be the case for ANG II and ET-1 in isolated afferent arterioles of Sprague-Dawley rats. Immediate O_2^- responses are blocked >90% by the NOX inhibitors apocynin and DPI and also by the cell-permeable scavenger Tempol (9, 10). Tempol has been observed to blunt a majority of ANG II-induced constriction of afferent arterioles of the isolated-perfused chronic hydronephrotic rat kidney (34).

ANG II causes rapid AT₁ receptor stimulation of PKC in descending vasa recta pericyte generation of ROS, with increases in cytosolic Ca²⁺ concentration and contraction in a Tempol- or DPI-sensitive manner (59). Thus there is a large body of evidence indicating that ANG II can rapidly stimulate NOX activity and O_2^- production in arteries, arterioles, and VSMC, and, importantly, the afferent arteriole, a major resistance vessel in the kidney, that are involved in Ca²⁺ signaling and contraction.

On the other hand, negative ex vivo effects of Tempol have been reported for artificially perfused renal vessels acutely stimulated by ANG II or NE (44, 54). Disparities in results may reflect different vascular preparations and experimental conditions. In vitro studies involving luminal perfusion with saline-like solutions should be interpreted with caution because these isolated preparations may overestimate the amounts and effects of NO since the perfusates are usually RBC-free or with a very low hematocrit and lacking physiological scavenging by oxy-hemoglobin.

O₂⁻-Mediated Catecholamine-Induced Renal Vasoconstriction

In addition to ANG II, our results document that O_2^- is involved in acute renal vasoconstriction elicited by intrarenal α -adrenoceptor stimulation by PE and NE. These G protein-coupled receptor agonists increase renal vascular resistance via a NOX-dependent mechanism in the presence as well as in the absence of NOS inhibition, indicating actions independent of NO availability. These characteristics for normotensive rats contrast with actions of NE in the chronic setting of hypertension, in which NE is considerably less efficacious than ANG II in upregulating vascular NOX activity and generating O_2^- (12, 14, 23).

Previous studies have found mixed results for involvement of O_2^- in the acute vascular effects of catecholamines. PE is known to rapidly stimulate O_2^- production in cultured aortic VSMC (2), and Tempol has been shown to blunt NE-induced constriction of afferent arterioles of the isolated-perfused, chronically hydronephrotic rat kidney (34). Moreover, both Tempol and SOD have been shown to blunt acute constriction of isolated mesenteric arteries and aortic rings contracted by NE (47, 57). However, one study reports that Tempol is ineffective in attenuating NE-induced acute constriction of isolated-perfused rabbit afferent arterioles (54). Clearly, further investigation is needed to investigate mechanisms in more detail to provide insight into possible differences between acute and chronic actions of various G protein-coupled receptor agonists on the microcirculation and the involvement and importance of ROS signaling in resistance arterioles.

O₂⁻-Mediated Renal Vasoconstriction Independent of NO

We found that the acute effects of both apocynin and Tempol on basal blood flow in the rat kidney were clearly evident in the absence of endogenous NO, if anything, tending to be greater than before NOS inhibition. Accordingly, the tonic constrictor influence of O_2^- on baseline RBF cannot be mediated by scavenging ambient NO and thus is probably due to a direct action of O_2^- on VSMC. Under basal conditions, O_2^- appears to be able to produce a direct constrictor action independent of NO in dog and rat kidneys (29, 31). Support for this view derives from the observation that inhibition of SOD

by intrarenal infusion of DETC causes a 20% reduction in RBF before and 40% reduction after NOS inhibition (29). The renal medullary effects of Tempol and SOD inhibition were similar to those we observed at the whole kidney level in that the constrictor effects of O_2^- on basal perfusion are independent of an interaction with NO (5). Other results indicate that the ability of Tempol to acutely blunt ANG II-induced constriction of dog kidneys was observed in the presence as well as in the absence of a NOS inhibitor (31). ANG II and ET-1 increase ROS, specifically O_2^- , in an apparent NO-dependent manner to increase cytosolic Ca^{2+} concentration in isolated afferent arterioles and pericytes of descending vasa recta (9, 10, 59). The SOD mimetic Tempol was found to normalize the degree of constriction of isolated-perfused rabbit afferent arterioles elicited by thromboxane mimetic U-46619 that was increased during NOS inhibition (42). Other investigators find that TGF-mediated responses are modulated in part by a constrictor action of O_2^- independent of the presence or absence of NO (26).

Our observation that superoxide causes agonist-induced acute renal vasoconstriction by a mechanism independent of NO is provocative, in contrast to the chronic effects of agonists in disease states characterized by endothelial dysfunction. At present, we do not know whether or not NO metabolism within the vasculature is affected by pharmacological agents during the acute experimental conditions of our studies. Our results would suggest not or that they are minimal. Previous studies examining the acute effects on ANG II on NO metabolism at the whole kidney level suggest intrarenal administration of apocynin or Tempol changes tissue levels of NO and urinary excretion of NO metabolites and 8-isoprostane (27, 31). However, it should be appreciated that, because of the preponderance of epithelial cells, tubular metabolic changes are likely to dominate at the whole kidney level. Consistent with this interpretation, NOX and ROS activities during basal conditions and during acute ANG II stimulation exert stronger effects on renal tubular transport of salt and water than on renal vascular resistance.

One interpretation of our results is that an increase in NOX activity in resistance arterioles generates O_2^- in a VSMC subcellular domain where Ca^{2+} signaling and contractility are relatively isolated from NO and its actions. Compartmentalization of NOX and SOD isoforms may be involved. Further studies are required to elucidate the mechanism(s) responsible for the acute vasoconstrictor effects of O_2^- independent of NO.

There are multiple mechanisms by which O_2^- can produce acute constriction of VSMC independent of NO. Most center on calcium homeostasis and signaling pathways such as Ca^{2+} entry via L-type Ca channels, inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from sarcoplasmic reticulum, activation of the ADP ribosyl cyclase/ryanodine receptor-mediated Ca^{2+} mobilization, and phosphorylation of myosin light chains (21, 28, 50, 51). Our laboratory has studied isolated renal afferent arterioles and found that ANG II or ET-1 rapidly induces production of O_2^- that leads to activation of the plasma membrane enzyme ADP ribosyl cyclase, resulting in increased sensitization of ryanodine receptors to mobilize Ca^{2+} from the sarcoplasmic reticulum, and thereby increases intracellular Ca^{2+} concentration (9, 10).

In summary, direct infusion of agents into the rat renal artery in vivo that inhibit NOX (apocynin) or enhance dismutation of

O_2^- (Tempol) produces mild but consistent vasodilation during basal conditions and marked impairment of vasoconstriction elicited by acute stimulation with ANG II, NE, or PE. Effects on baseline and vasoconstrictor responses were both found to be independent of NO. We conclude that ROS, in particular, O_2^- , play an important role in the normal regulation of renal function as evidenced by tonic constrictor action of the renal microcirculation under basal conditions and in acute renal vascular reactivity to G protein-coupled receptor stimulation. These constrictor effects of O_2^- do not require NO. Thus, in healthy kidneys, O_2^- exerts rapid and direct effects on the renal vasculature, in addition to inactivation of macula densa- or endothelium-derived NO, extending those previously described for TGF and under pathophysiological conditions (e.g., hypertension and diabetic nephropathy). In the acute setting in the renal circulation, the contribution of O_2^- to acute constrictor responses is not limited to ANG II but also includes α -adrenergic responses to NE and PE.

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