Oxidant stress in kidneys of spontaneously hypertensive rats involves both oxidase overexpression and loss of extracellular superoxide dismutase

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Adler, S., and H. Huang. Oxidant stress in kidneys of spontaneously hypertensive rats involves both oxidase overexpression and loss of extracellular superoxide dismutase. Am J Physiol Renal Physiol 287: F907–F913, 2004; doi:10.1152/ajprenal.00060.2004.—Oxidant stress is an important contributor to renal dysfunction and hypertension. We have previously demonstrated that regulation of renal oxygen consumption by nitric oxide (NO) is impaired in the kidney of spontaneously hypertensive rats (SHR) due to increased superoxide production. We further explored the mechanisms of enhanced oxidant stress in the kidney of SHR. Suppression of cortical oxygen consumption by bradykinin (BK) or enalaprilat (Enal), which act through stimulation of endogenous NO, was impaired in SHR (BK: −14.1 ± 1.2%; Enal: −15.5 ± 1.2%) and was restored by addition of apocynin, an inhibitor of assembly of the NAD(P)H oxidase complex (BK: −21.0 ± 0.6%; Enal: −25.3 ± 1.4%), suggesting this as the source of enhanced superoxide production. Addition of an angiotensin type 1 receptor blocker, losartan, also restored responsiveness to control levels (BK: −22.0 ± 1.1%; Enal: −23.6 ± 1.3%), suggesting that ANG II is responsible for enhanced oxidase activity. A similar defect in responsiveness to BK and Enal could be induced in Wistar-Kyoto kidneys by ANG II and was reversed by a superoxide scavenger (tempol), apocynin or losartan. Immunoblotting of cortical samples demonstrated enhanced expression of endothelial NO synthase (eNOS) (1.9×) and NAD(P)H oxidase components (gp91phox 1.6× and Rac-1 4.5×). Expression of SOD-1 and −2 were unchanged, but SOD-3 was significantly decreased in SHR (0.5×). Thus NO bioavailability is impaired in SHR owing to an ANG II-mediated increase in superoxide production in association with enhanced expression of NAD(P)H oxidase components, despite increased expression of eNOS. Loss of SOD-3, an important superoxide scavenger, may also contribute to enhanced oxidant stress.

ENHANCED LEVELS OF OXYGEN radicals, leading to a state of oxidant stress, have been shown to be present in many disease states characterized by endothelial dysfunction and vascular injury, including diabetes, hypertension, and chronic renal insufficiency, both in animal models and in humans (14, 15, 22, 26–28, 37). The rapid interaction of superoxide with nitric oxide (NO) results in the production of peroxynitrite, another harmful radical, and the destruction of NO, decreasing NO bioavailability in situations of oxidant stress (10, 25). Decreased availability of NO, resulting in vasoconstriction and loss of endothelium-mediated vasodilation, has been documented in models of hypertension and in human vessels (12, 20, 28, 35). In particular, in the spontaneously hypertensive rat (SHR) model, the contribution of oxidant stress to the development of hypertension has been suggested in studies demonstrating amelioration or reversal of hypertension by the use of oxygen radical scavengers (27, 28, 37), and we have demonstrated a decrease in NO bioavailability due to superoxide in the kidneys of SHR (2). Elevated blood pressure, in and of itself, may also contribute to superoxide production via activation of vascular NAD(P)H oxidase (31), leading to a vicious cycle that perpetuates hypertension.

Levels of oxidant stress are determined by the rates of oxygen radical production and scavenging. The discovery of homologs of the phagocyte NADPH oxidase that are expressed in blood vessels and organs such as the kidney has allowed further characterization and a better understanding of the sources of oxygen radicals other than white blood cells (8, 9, 19, 29). ANG II has been found to be a major stimulus for oxygen radical production via stimulation of these oxidases, defining another mechanism for its injurious effects (19, 23). Possibly of equal importance in the control of oxidant stress and nitric oxide bioavailability are oxygen radical scavenging mechanisms, such as the superoxide dismutases, which also play an important role in ameliorating hypertension (4, 17).

We have previously shown that there is impaired regulation of renal O2 consumption in SHR in response to stimulators of NO production (2). Experiments with the superoxide scavenger tempol suggested that increased superoxide levels lead to decreased NO bioavailability in these animals (2). Other studies have suggested vascular and renal oxidases as sources of enhanced oxidant stress in hypertension and other disease states (9, 19, 23). We hypothesized that the enhanced oxidant stress in these animals, leading to decreased NO and possibly potentiating hypertension, was due to enhanced oxygen radical production by the NAD(P)H oxidase complex or decreased scavenging of superoxide by endogenous systems. We also speculated that ANG II played a role in the development of oxidant stress. The studies presented here tested these possibilities in isolated kidney tissue from SHR.

MATERIALS AND METHODS

Reagents. Bradykinin, enalaprilat, S-nitroso-N-acetylpenicillamine (SNAP), N-nitro-l-arginine methyl ester (l-NAME), 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (tempol), sodium succinate, and sodium cyanide were purchased from Sigma (St. Louis, MO). 4-Hydroxy-3-methoxyacetophenone (apocynin) was purchased from Fluka (Sigma). Losartan (DuP 753) was a gift from DuPont Pharmaceutical (Wilmington, DE). Affinity-purified monoclonal mouse antibodies to endothelial nitric oxide synthase (eNOS/NOS type III), manganese (MnSOD; SOD-2), and gp91phox were purchased from BD Transduction Laboratories (San Diego, CA). Affinity-puri-

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fied polyclonal antibody (from rabbit) to Rac-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody (from sheep) to Cu/Zn SOD (SOD-1) was purchased from Calbiochem (La Jolla, CA). Affinity-purified polyclonal antibody (from rabbit) to extracellular SOD (ecSOD; SOD-3) was a gift from Dr. Wei Wang (Univ. of Colorado Health Sciences Center, Denver, CO). Donkey anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Sigma.

**Animals.** Male SHR and Wistar-Kyoto rats (WKY) were purchased from Taconic Farms (Germantown, NY) at 10 wk of age and studied after 1 wk of acclimatization. Rats were maintained on a standard rat chow with 0.4% sodium content (Laboratory Rodent Diet, Richmond, IN) and allowed free access to food and water until the day of study. After death, the left kidneys were removed, decapsulated, and weighed. Tissue samples from the right kidney cortex were snap-frozen in liquid nitrogen and stored at −80°C for measurement of eNOS, SOD-1, -2, and -3, gp91phox, and Rac-1 levels (see below). The protocols used conformed to the **Guiding Principles for the Care and Use of Laboratory Animals** of the American Physiological Society and the National Institutes of Health.

**Preparation of kidney tissue slices and measurement of O2 consumption.** Thin slices of renal cortex (−1 mm, weight 10−20 mg) were prepared and incubated in Krebs bicarbonate solution containing (in mmol/l) 118 NaCl, 4.7 KCl, 1.5 CaCl2, 25 NaHCO3, 1.2 KH2PO4, 1.1 MgSO4, and 5.6 glucose, pH 7.4, bubbled with 21% O2-5% CO2-74% N2 at 37°C for 2 h. At the end of incubation, each piece of tissue was placed in a stirred chamber with 3 ml of air-saturated Krebs bicarbonate solution containing 10 mmol/l HEPES and 5.6 mmol/l glucose (pH 7.4). The chamber was sealed with a Clark-type platinum O2 electrode (Yellow Springs Instruments, Yellow Springs, OH). O2 consumption was measured polarographically using an O2 monitor (model YSI 5300) connected to a linear chart recorder (model 1202, Barnstead/Thermolyne, Dubuque, IA). Dose-response curves of the effect of different agonists on kidney O2 consumption were then measured. Succinate (10−3 mol/l) and then sodium cyanide (10−3 mol/l) were added at the end of each experiment to confirm that changes in O2 consumption originated from mitochondrial respiration.

Renal cortical O2 consumption was calculated as the rate of decrease in O2 concentration, assuming an initial O2 concentration of 224 nmol/ml (calculated from O2 solubility at 37°C and 1 atm), and is expressed as nanomoles O2 consumed per minute per gram of tissue. O2 consumption due to the electrode is <5% of that observed in the presence of tissue. The effects of drugs used on O2 consumption are expressed as percent change from baseline O2 consumption. Baseline O2 consumption was measured in the cortex in the absence and presence of l-NAME (10−3 mol/l) in each group.

**Effect of agonists of NO production on O2 consumption.** Bradykinin or enalaprilat at concentrations of 10−7−10−4 mol/l was added in a cumulative concentration-dependent manner. They were used to measure the effects of stimulation of endogenous NO production on renal O2 uptake. The response to these drugs was also examined after preincubation with L-NAME (10−3 mol/l). Each condition was tested in six or seven rats from each group.

**Immunoblotting of proteins.** Samples of renal cortex were pulverized in liquid nitrogen and homogenized in 5 vol of lysis buffer (0.05 M Tris-HCl, pH 7.2, 1 mM EDTA, 0.01 M diithiothreitol, 1 mg/ml PMSF, 100 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor, and 20 μg/ml aprotinin, volume = 5× tissue weight) followed by sonication for 1 min at 4°C. Lysates were centrifuged at 10,000 g for 10 min at 4°C and stored at −80°C until use. Protein content of supernatants was measured using a Bio-Rad protein assay (Bio-Rad Laboratories).

Samples of tissue lysate (100 μg of protein) were loaded into individual lanes, subjected to electrophoresis on 8 or 15% polyacrylamide gels, and electrophoretically transferred from the gels to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) using a semidyfer transfer cell (Bio-Rad). After at least 1 h of blocking with 5% milk/PBS, membranes were incubated with antibodies to eNOS, SOD-1, SOD-2, SOD-3, gp91phox, or Rac-1 in 1% milk/PBS at 4°C overnight. After being washed, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies to mouse, rabbit, or sheep IgG in 1% milk/PBS at room temperature for 2 h. Membranes were also probed with antibody to β-actin (Novus Biologicals, Littleton, CO) to correct for differences in protein loading. Sites of antibody-antigen reaction were observed using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) before exposure to X-ray film (Kodak, Rochester, NY).

The relative intensities of bands in autoradiograms were determined on an AlphaInmager 2000 documentation and analysis system (Alpha Innotech, San Leandro, CA) followed by analysis using image software. Band intensity for each measured protein was divided by the intensity of the corresponding β-actin band to correct for any differences in protein loading.

**Statistical analysis.** All data are expressed as means ± SE. Statistical analysis of baseline O2 consumption and densities of protein bands was performed using Student’s t-test. Changes in O2 consumption caused by drug treatment were analyzed using two-way ANOVA followed by multiple comparisons using the Tukey test (Sigma-Stat, SPSS-Science, Chicago, IL). Statistical significance was achieved at P < 0.05.

**RESULTS**

**Baseline renal cortical O2 consumption.** Baseline renal cortical O2 consumption was similar in the two groups, in agreement with our previously published results (2): WKY: 1.211 ± 87 nmol O2·min−1·g−1, n = 6; SHR: 1.195 ± 117 nmol O2·min−1·g−1, n = 7, P > 0.05. Addition of the NOS inhibitor l-NAME (10−3 mol/l) did not significantly alter O2 consumption (WKY: 1.235 ± 126 nmol O2·min−1·g−1, n = 6; SHR: 1.243 ± 90 nmol O2·min−1·g−1, n = 7, P > 0.05).

**Effect of bradykinin and enalaprilat on renal cortical O2 consumption.** Cumulative doses of bradykinin (10−7−10−4 mol/l) produced significant, concentration-dependent decreases in renal cortical O2 consumption in WKY and SHR rats (WKY: from −1.3 ± 0.9 to −25.3 ± 2.3%, n = 6; SHR: from −0.3 ± 0.3 to −14.1 ± 1.2%, n = 7). The depression of renal cortical O2 consumption by bradykinin was significantly less in SHR than WKY rats (P < 0.05), confirming previous observations (2) (Fig. 1A). Similarly, the angiotensin-converting enzyme inhibitor enalaprilat (10−7−10−4 mol/l), which also stimulates endogenous NO production, caused concentration-dependent decreases in renal cortical O2 consumption in WKY and SHR rats (WKY: from −1.0 ± 0.6 to −25.6 ± 1.4%, n = 6; SHR: from 0 ± 0 to −15.5 ± 1.2%, n = 7), with a significantly lower effect in SHR than WKY rats (P < 0.05)
O2 consumption in SHR, we explored the source of increased ability of bradykinin and enalaprilat to suppress renal cortical superoxide scavenging of superoxide with tempol had restored the superoxide production, we explored the effect of blocking because ANG II has been implicated in increases in ANG II action with the AT1-R antagonist losartan. Renal cortical tissue was incubated with losartan (10^-6 mol/l) before performance of dose-response curves. Inhibition of renal cortical O2 consumption by bradykinin and enalaprilat was significantly augmented by losartan in SHR but not WKY rats (Fig. 3). In the presence of losartan, the response in SHR was not significantly different from that in WKY animals (bradykinin+losartan in WKY: from -1.4 ± 0.7 to -23.5 ± 1.6%, n = 6, SHR: from 0 ± 0 to -22.0 ± 1.1%, n = 7, P > 0.05; enalaprilat+losartan in WKY: from -2.0 ± 0.7 to -24.6 ± 1.5%, n = 6, SHR: from -0.7 ± 0.5 to -23.6 ± 1.3%, n = 7, P > 0.05).

Effect of NO donor SNAP on renal cortical O2 consumption. Administration of cumulative doses of the NO donor SNAP (10^-7-10^-4 mol/l) decreased renal cortical O2 consumption similarly in WKY and SHR, demonstrating no inherent differences between the strains in responsiveness of O2 consumption to exogenous NO (Fig. 4). Addition of tempol (data not shown), apocynin, losartan, or L-NAME did not affect responsiveness to SNAP.

Effect of ANG II on renal cortical O2 consumption. Because experiments with losartan suggested ANG II as a cause of increased superoxide production in SHR, we explored the effect of ANG II on renal cortical O2 consumption in WKY rats. Preincubation of renal cortical tissue from WKY rats with ANG II (10^-8 mol/l) significantly suppressed responsiveness to inhibition of renal cortical O2 consumption by bradykinin (bradykinin: from 0 ± 0 to -23.5 ± 1.0%, n = 6; bradykinin+losartan: from -1.4 ± 0.7 to -23.5 ± 1.6%, n = 6).

Effect of apocynin on renal cortical O2 consumption. Because scavenging of superoxide with tempol had restored the ability of bradykinin and enalaprilat to suppress renal cortical O2 consumption in SHR, we explored the source of increased superoxide production in these animals using apocynin, an inhibitor of NAD(P)H oxidase assembly. Bradykinin and enalaprilat both decreased O2 consumption significantly more in WKY rats than in SHR (Fig. 2). Addition of apocynin significantly augmented the suppression of O2 consumption by bradykinin (Fig. 2A) and enalaprilat (Fig. 2B) in SHR rats to a level that was not different from that in WKY rats (bradykinin+apocynin in WKY: from -0.0 ± 0.0 to -22.6 ± 1.7%, n = 6, SHR: from -0.6 ± 0.6 to -21.0 ± 0.6%, n = 7, P > 0.05; enalaprilat+apocynin in WKY: from -1.2 ± 0.7 to -24.6 ± 1.2%, n = 6, SHR: from -0.9 ± 0.7 to -25.3 ± 1.4%, n = 7, P > 0.05).

Effect of angiotensin blockade on renal cortical O2 consumption. Because ANG II has been implicated in increases in superoxide production, we explored the effect of blocking

Fig. 1. Effect of cumulative doses of bradykinin (A) or enalaprilat (B) on renal cortical O2 consumption in Wistar-Kyoto (WKY; ●) and spontaneously hypertensive rats (SHR; ▲). Both drugs caused significant dose-dependent decreases in O2 consumption that were significantly less in SHR. *P < 0.05 vs. WKY rats.

Fig. 2. Effect of inhibition of NAD(P)H oxidase with apocynin on inhibition of renal cortical O2 consumption by cumulative doses of bradykinin (A) or enalaprilat (B). The response to both drugs was significantly less in SHR (▲) than in WKY (●) rats. Addition of apocynin had no effect in WKY rats (□) but restored responsiveness in SHR (△). *P < 0.05 vs. SHR with apocynin.
DISCUSSION

The studies presented here confirm our previous work in the SHR kidney (2), namely, of decreased NO bioavailability, reflected as an impairment of regulation of renal $O_2$ consumption, and again suggest excess superoxide production as the cause. We have used the regulation of renal oxygen consumption by NO as a direct measure of a NO effect in renal cortex in vitro in the absence of glomerular filtration, thus demonstrating a basic change in renal metabolism in SHR independent of the workload of sodium transport. Decreased NO bioavailability occurs despite increased expression of eNOS, the major NOS isoform responsible for regulation of renal oxygen consumption by NO (1). The data also suggest that the major source of superoxide is the NAD(P)H oxidase complex and that ANG II plays an important role in the stimulation of superoxide production. Finally, we document that in addition to increased expression of proteins involved in the assembly of the NAD(P)H oxidase complex in the kidney, there is loss of an important antioxidant defense, extracellular SOD (SOD-3). The end result of these processes is an increase in the level of oxidant stress mediated mainly through an effect of ANG II.

Enhanced production of superoxide has been demonstrated in numerous models of hypertension, including those due to partial nephrectomy, lead-induced hypertension, salt-sensitive hypertension, and the SHR (2, 14, 20, 21, 26–28, 35, 37). Evidence of enhanced oxidative stress has been obtained in

Levels of renal cortical proteins (NOS, SOD, gp91phox, Rac-1). Expression of several proteins involved in NO production and metabolism of oxygen radicals were measured in samples of renal cortex from WKY and SHR rats (Fig. 6). Despite evidence of decreased NO bioavailability in SHR, levels of eNOS protein in renal cortex of SHR are significantly increased compared with WKY rats (SHR: 3.02 ± 0.53, n = 7; WKY: 1.58 ± 0.07, n = 6, P < 0.05). A similar, albeit smaller, increase was seen in medullary eNOS (data not shown). Levels of SOD-1 and SOD-2 were similar in both groups of rats (SOD-1: SHR: 0.44 ± 0.08, n = 7; WKY: 0.59 ± 0.08, n = 7, P > 0.05; SOD-2: SHR: 0.42 ± 0.12, n = 6; WKY: 0.42 ± 0.05, n = 6, P > 0.05), but levels of extracellular SOD (SOD-3) were significantly reduced in SHR (SHR: 0.32 ± 0.04, n = 7; WKY: 0.62 ± 0.08, n = 7, P < 0.01). Two components of the NAD(P)H oxidase complex, gp91phox and Rac-1, are both significantly increased in SHR rats (gp91phox: SHR: 3.13 ± 0.49, n = 7; WKY: 2.0 ± 0.09, n = 7, P < 0.05; Rac-1: SHR 1.38 ± 0.28, n = 7; WKY: 0.31 ± 0.05, P < 0.01).

Levels of renal cortical O2 consumption by cumulative doses of bradykinin (Fig. 3A). Scavenging of superoxide with tempol, inhibition of NAD(P)H oxidase with apocynin, or blockade of the AT1 R with losartan all restored the responsiveness to inhibition of renal cortical O2 consumption by bradykinin toward the SHR (Fig. 5B). The response to both drugs was significantly less in SHR (Fig. 3B). The data suggest that the major source of superoxide is the NAD(P)H oxidase complex and that ANG II plays an important role in the stimulation of superoxide production. Finally, we document that in addition to increased expression of proteins involved in the assembly of the NAD(P)H oxidase complex in the kidney, there is loss of an important antioxidant defense, extracellular SOD (SOD-3). The end result of these processes is an increase in the level of oxidant stress mediated mainly through an effect of ANG II.

Enhanced production of superoxide has been demonstrated in numerous models of hypertension, including those due to partial nephrectomy, lead-induced hypertension, salt-sensitive hypertension, and the SHR (2, 14, 20, 21, 26–28, 35, 37). Evidence of enhanced oxidative stress has been obtained in cats treated with phenylthiourea (20, 21), in rats with partial nephrectomy (35), and in rats with lead-induced hypertension (37). The studies presented here confirm our previous work in the SHR kidney (2), namely, of decreased NO bioavailability, reflected as an impairment of regulation of renal $O_2$ consumption, and again suggest excess superoxide production as the cause. We have used the regulation of renal oxygen consumption by NO as a direct measure of a NO effect in renal cortex in vitro in the absence of glomerular filtration, thus demonstrating a basic change in renal metabolism in SHR independent of the workload of sodium transport. Decreased NO bioavailability occurs despite increased expression of eNOS, the major NOS isoform responsible for regulation of renal oxygen consumption by NO (1). The data also suggest that the major source of superoxide is the NAD(P)H oxidase complex and that ANG II plays an important role in the stimulation of superoxide production. Finally, we document that in addition to increased expression of proteins involved in the assembly of the NAD(P)H oxidase complex in the kidney, there is loss of an important antioxidant defense, extracellular SOD (SOD-3). The end result of these processes is an increase in the level of oxidant stress mediated mainly through an effect of ANG II.
Evidence that scavenging of NO by superoxide contributes to hypertension comes from experiments demonstrating increased NO bioavailability and improved endothelium-mediated vasorelaxation via NO. Similarly, Guzik et al. (11) demonstrated superoxide production in all three vessel layers (adventitia, media, endothelium) but also noted that NO production scavenges superoxide, suggesting a much more complex interaction within the vessel wall.

Evidence that scavenging of NO by superoxide contributes to hypertension comes from experiments demonstrating increased NO bioavailability and decreased blood pressure after manipulations that decrease oxygen radical levels. Administration of superoxide scavengers ameliorates hypertension in SHR and salt-sensitive hypertension (21, 27, 28, 37). Production of superoxide in the renal cortex and medulla in Dahl salt-sensitive rats was decreased by tempol in association with a drop in blood pressure (21). Life-long supplementation with antioxidants also delays the onset of hypertension, as well as ameliorating its severity, in SHR (42), along with decreasing markers of oxidative stress. Directly decreasing production of superoxide with a highly specific inhibitor of vascular NAD(P)H oxidase decreases blood pressure in a mouse model of hypertension induced by ANG II infusion (24). Finally, scavenging of superoxide by SOD leads to decreases in blood pressure. This is demonstrated by significantly increased blood pressure in SOD-3-deficient mice with one clipped kidney or after infusion with ANG II compared with controls. (17).

Infusion of recombinant SOD-3 into ANG II-treated mice rapidly lowered blood pressure (17). Transfection of SHR with the gene for human SOD-3 similarly lowered blood pressure (4). This was accompanied by improvement of acetylcholine-mediated vascular relaxation, suggesting increased availability of endothelium-generated NO.

There are several sources of increased superoxide production potentially implicated in vascular pathology, including xanthine oxidase, cytochrome P-450, uncoupled NOS, and the NAD(P)H oxidases (19). A kidney specific oxidase (Renox or NOX-4) has been described but is also found in vascular smooth muscle cells (8, 13, 29). Previous work in the SHR has documented overexpression of several components of the NAD(P)H oxidase complex through measurement of mRNA and/or protein levels by immunoblotting (3, 30, 42). Chabrashvili et al. (3) found expression of the p22(phox), p47(phox), and p67(phox) subunits of the NAD(P)H complex in the renal vasculature and elements of the distal tubule (starting at the thick ascending limb) in the kidney of SHR with significantly increased levels of p47- and p67-phox in SHR compared with WKY animals. Zhan et al. (42) described increased levels of gp91(phox), the catalytic subunit of the complex, and p22(phox) in the renal cortex of SHR. In the latter studies, the feeding of antioxidants not only reduced hypertension and oxidative stress but also decreased the level of expression of these subunits. Our work confirms overexpression of gp91(phox) and the small G protein Rac-1 that is necessary for activation of the oxidase. In thoracic aortas of SHR, increased levels of p22(phox) were also detected (30). Overexpression of these components, along with...
the beneficial effects of apocynin, an inhibitor of assembly of the complex, on the responsiveness of \( \text{O}_2 \) consumption to stimulators of NO production, suggests that the NAD(P)H complex is an important source of oxygen radicals in SHR.

Another potential source of superoxide is uncoupled NOS. When levels of arginine or tetrahydrobiopterin (BH\(_4\)) are low, NOS activation may favor production of superoxide rather than NO (32, 41). Evidence of enhanced oxygen radical production by NOS has been found in aortas from young, prehypertensive SHR (5), and administration of BH\(_4\) to SHR animals suppresses the development of hypertension as well as decreases superoxide production by aortic segments (16). Levels of NOS isoforms in the kidney have been reported to be elevated in SHR, including inducible NOS, neuronal NOS, and eNOS (33, 40). We have confirmed elevated eNOS expression in the renal cortex of SHR but have been unable to detect either inducible NOS or neuronal NOS in cortical samples. While we have not directly studied the possibility that uncoupled NOS in the renal cortex leads to the observed increase in oxidant stress, the ability of apocynin to completely reverse the defect seen in SHR suggests that NAD(P)H oxidase is the most important source of superoxide in the kidney.

The ability of the AT\(_1\)R antagonist losartan to restore the effect of bradykinin and enalaprilat on the regulation of renal \( \text{O}_2 \) consumption suggests a role of ANG II in mediating enhanced oxidant stress in SHR. This is further supported by the observation of induction of a similar defect in kidney from WKY rats by incubation with ANG II, a defect again reversed by superoxide scavenging with tempol or inhibition of NAD(P)H oxidase assembly with apocynin. ANG II is known to stimulate both activity and expression of NAD(P)H oxidase (9, 13, 19), which in turn reduces NO availability through scavenging. \( \text{O}_2 \) utilization in the kidney of SHR for sodium transport has been shown to be less efficient than in WKY rats (38), an observation consistent with our finding of impaired regulation of renal \( \text{O}_2 \) consumption in SHR and decreased NO bioavailability (2). This defect is largely reversed by the AT\(_1\)R blocker candesartan in vivo (39), suggesting that activation of the AT\(_1\)R mediates the increased \( \text{O}_2 \) consumption. Our data further support a role in vitro for an AT\(_1\)R-mediated increase in renal oxidant stress, leading to decreased NO bioavailability in the SHR.

Of interest, we also found that levels of extracellular SOD were reduced by \( \sim 50\% \) in SHR, potentially contributing further to oxidant stress in these animals. Studies in mice lacking either SOD-3 or Cu/Zn SOD (SOD-2) have suggested that deficiency of either of these enzymes can lead to increased superoxide levels and loss of NO, leading to decreased endothelium-mediated vasodilatation (6, 17), although basal blood pressure was not elevated in either group of deficient animals. Under stress, induced by clipping of one kidney or ANG II infusion, blood pressure was higher in SOD-3-deficient animals (17). Overexpression of human SOD-3 in SHR also leads to lowering of blood pressure (4), further supporting the importance of superoxide scavenging mechanisms in responding to oxidant stress. Another study of SOD expression in the aorta of SHR found increased levels of SOD-1 and SOD-2, whereas SOD-3 was not measured (30). These animals were, however, much older than the ones studied here, and these results might represent a change with aging or different regulation of SODs in the vessel wall vs. the kidney. Because expression of SOD-3 in mice is regulated by NO, the loss of SOD-3 in SHR may be a secondary, albeit aggravating, factor (7). In other models with enhanced oxidant stress, namely % nephrectomy, salt loading, and lead-induced hypertension, renal expression of SOD-1 has been found to be decreased or increased, whereas SOD-2 was decreased or unchanged (18, 34, 36). Thus a general pattern of response of SOD expression in situations of oxidant stress is not apparent.

In summary, we have further confirmed the role of enhanced intrarenal superoxide production in limiting NO availability in the kidney in the SHR model of hypertension. We have demonstrated that enhanced expression and activation of elements of the NAD(P)H complex occur in these animals, contributing to oxidant stress, along with loss of an important counterregulatory element, SOD-3. Loss of NO occurs despite enhanced expression of eNOS, which in itself could be a response to lower levels of NO. Finally, an important role of stimulation of the AT1R by ANG II in the enhanced oxidant stress that occurs in these animals has been demonstrated.

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