Role of Angiotensin II and Free Radicals in Blood Pressure Regulation in a Rat Model of Renal Hypertension

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Abstract—One-kidney, 1-clip rats (1K1C) or uninephrectomized controls were treated with either the superoxide dismutase mimetic tempol (0.5 mmol · kg⁻¹ · d⁻¹), angiotensin type 1 receptor inhibitor losartan (50 mmol · L⁻¹ · kg⁻¹ · d⁻¹), or both (n=6 per group) for 2 weeks. At the end of the study, systolic blood pressure (BP) decreased on average by 21% in tempol-treated and 29% in losartan-treated versus untreated 1K1C (217±4.4 mm Hg) and was normalized in the losartan plus tempol group. Mean BP also decreased from 159±3.7 mm Hg in 1K1C to 93±2.8 mm Hg in the losartan plus tempol group. Also, aortic wall area was reduced by 18% in losartan- or tempol-treated 1K1C and by 30% in losartan plus tempol rats compared with untreated 1K1C. Plasma renin activity was increased from 4.8±0.3 in untreated 1K1C to 15.9±0.9 ng · mL⁻¹ · h⁻¹ in losartan-treated but not tempol-treated 1K1C. Superoxide generation by the isolated aortic rings assessed by lucigenin chemiluminescence was significantly decreased (by ~40%) in all losartan, tempol, and losartan plus tempol groups compared with untreated 1K1C. Nitrotyrosine ELISA in the kidney displayed a significant reduction, from 59±13 ng/mg of protein in 1K1C to 12.5±5 ng/mg of protein in the losartan plus tempol 1K1C. Western blotting for nNOS in kidney cortex and medulla showed a protein increase in both fractions of 1K1C versus controls and was normalized by losartan plus tempol treatment. Collectively, data show a synergistic effect of losartan and tempol on BP reduction in 1K1C rats. The mechanism may involve reduced superoxide production and nitrotyrosine formation in kidney and decreased kidney neuronal-type NO synthase expression in treated animals. This status in the oxidative balance seems to affect BP in the renal hypertensive rats. (Hypertension. 2001;38:361-366.)

Key Words: superoxide ■ nitric oxide ■ nitrotyrosine ■ losartan ■ tempol ■ kidney ■ aorta

Involvement of oxidative stress in the pathology of hypertension was reported recently for humans with essential hypertension,1,2 preeclamptic human women,3 and animal models such as the spontaneously hypertensive,4,5 Dahl salt-sensitive,6,7 or angiotensin (Ang) II–infused rat.8 In addition, an important role of free radicals in blood pressure (BP) regulation was shown in a model of lead-induced hypertension,9 in chronic renal failure10 and in a model of diet-induced hypertension in rat.11 Moreover, a direct role of oxidative stress in inducing hypertension was shown by Vaziri et al.12 in intact genetically normotensive rats that were made hypertensive by direct induction of oxidative stress via in vivo glutathione depletion. The main free radical that seems to be involved in the pathology of different forms of hypertension is the superoxide anion. This can either act as a vasoconstrictor13,14 or, under certain circumstances, can interact rapidly with NO15 to reduce its bioavailability and further increase vasoconstriction.16,17 Furthermore, peroxynitrite formed after interaction of superoxide with NO is a more potent oxidant that can nitrosylate tyrosine residues in proteins,18 therefore potentially altering their function. Treatment of hypertensive rats with either liposome-encapsulated superoxide dismutase (SOD)8 or SOD mimetics5,19 was able to reduce BP and superoxide anion production significantly. The major source of superoxide in the vascular wall seems to be the NADH/NADPH system.20,21 Interestingly, in vitro studies showed that activity of the enzyme seems to be regulated by both Ang II22,23 and cyclical stretch.24,25 However, it is not clear whether in vivo Ang II or pressure or both have a role in the involvement of oxidative stress in BP regulation. Laursen et al.8 showed in a model of Ang II infusion in rat that Ang II and not pressure is involved in superoxide production and indirectly in BP regulation. Nevertheless, the level of angiotensin in this model is apparently far higher than in other forms of hypertension in experimental models and humans. Recently, Reckelhoff et al.26 showed that even low suppressor doses of Ang II are able to generate oxidative stress in vivo and induce a chronic increase in BP. The purpose of the present study was to investigate in vivo the involvement of oxidative stress in BP regulation in the 1-kidney, 1-clip (1K1C) hypertensive rat. Similar to some forms of human essential hypertension, the 1K1C model of renal hypertension in rat is not dependent on the renin-angiotensin system, at least starting from week 2 after surgery.27 Therefore, blocking the angiotensin type 1 (AT1) receptor with losartan will leave BP virtually unchanged.28 Rats were treated with losartan for 2 weeks to
delineate involvement of Ang II through the AT1 receptor in oxidative stress and with tempol to assess involvement of superoxide anion. Tempol, a SOD mimetic permeable for cell membrane, was shown to lower BP efficiently in spontaneously hypertensive but not Wistar-Kyoto rats.

Materials

Treatment Groups
Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, Ind), were randomly divided into 2 groups; uninephrectomized controls (n=32) and 1K1C hypertensives (n=32). Surgery was performed as described by Brooks et al. After a 2-day recovery, rats were further subdivided in 8 groups (n=8 per group): 1K1C and controls given vehicle, tempol, losartan, or combined tempol and losartan. Both tempol and losartan were administered daily in drinking water (0.34 g/L [≈0.5 mmol · kg−1 · d−1] tempol and 0.2 g/L [≈50 mmol · kg−1 · d−1] losartan) for 2 weeks. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Eastern Virginia Medical School.

Blood Pressure
Systolic BP was measured under conscious conditions by use of the tail-cuff method with a Narco Biosystems electrophysgmonanometer. Before rats were killed, mean BP was measured by cannulation of the tail artery using a Brush transducer and model 2200 recorder (Gould). Rats were killed, blood was collected on ice on 0.01% EDTA, and aorta and kidneys were collected and either used immediately or snap-frozen in liquid nitrogen.

Morphological Analysis
Internal and external circumferences of each toluidine blue–stained vessel section were measured with a video-based image system with edge-tracking software (JAVA, Jandel Scientific). Mean of 3 different measurements was used to calculate intimal-medial area.

Superoxide anion production was measured in isolated aortic rings by use of a method previously described, with 25 μmol/L lucigenin. Integrated readings over 15 minutes were normalized to milligrams tissue protein measured by BCA method (Sigma kit).

Western Blotting
After residual blood was removed by PBS perfusion, kidney cortex and medulla were homogenized in Tris-HCl buffer pH 6.8 containing 1 mmol/L EDTA, 1% SDS, 10% glycerol, 50 mmol/L NaF, 10 μg/mL leupeptin, 20 μg/mL aprotinin, and 1 mmol/L PMSF, final concentrations. After 8% polyacrylamide gel electrophoresis in nonreducing conditions and electroblotting, membranes were reacted with a monoclonal antibody for neuronal-type NO synthase (nNOS; type I) isoform (Transduction Laboratories) at a 1:2500 dilution, with a monoclonal antibody for nitrotyrosine and reagents of the sample.

Enzyme immunoassay with a monoclonal antibody for nitrotyrosine and reagents of the sample.

Figure 1. Systolic (A) and mean (B) arterial pressure in 1K1C and control rats treated with tempol (0.5 mmol · kg−1 · d−1), losartan (50 mmol · L−1 · kg−1 · d−1), or both tempol and losartan for 2 weeks. C, Correlation between mean and systolic BP in rats from all 6 study groups (n=46). Data represent mean±SE of 8 animals per group. *P<0.05 vs control.

Statistics
Data are shown as mean±SE. To determine significance (P<0.05) between different groups, 1-way ANOVA followed by Tukey’s post hoc test was performed.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results
Systolic BP measured by tail-cuff method was significantly increased in 1K1C rats from day 3 after surgery until the end of the experiment, in agreement with data reported previously. When 1K1C rats were treated with the SOD mimetic tempol (0.5 mmol · kg−1 · d−1) for 2 weeks, systolic BP decreased from an average of 212.5±9.3 mm Hg to an average of 166±6.9 mm Hg, and mean arterial pressure dropped by 13% to an average value of 138.4±8.0 mm Hg (Figure 1A and 1B). When rats were given the AT1 receptor inhibitor losartan (50 mmol · L−1 · kg−1 · d−1), a reduction of
systolic BP to 170.0±13.0 mm Hg was observed, paralleled by a 12% reduction in mean arterial pressure. After 2 weeks of treatment with both losartan and tempol, systolic BP in 1K1C rats was substantially reduced by 40%, and mean BP also dropped to 104.2±2 mm Hg, close to BP in the control group (Figure 1A and 1B). In control groups treated with losartan, tempol, or both, BP was not changed significantly compared with that of untreated controls (Figure 1A and 1B). As shown in Figure 1C, very good correlation exists between systolic and mean BP (r=0.9098, P<0.001) for individual rats in each experimental group, which suggests that diastolic BP varies proportionally and in the same direction as systolic BP. Therefore, this provides indirect evidence that treatment with losartan and tempol also can reduce diastolic BP significantly. We showed before that in 1K1C rats, hypertension is associated with vascular hypertrophy in both large and small arteries.32 To examine the effect of losartan and tempol on arterial hypertrophy, we measured the cross-sectional area of the thoracic aorta in all experimental groups; results are illustrated in Figure 2A. Both losartan and tempol individually and in combination were proven to be efficient for reduction of aortic wall area in 1K1C rats by 22% to 30% compared with untreated 1K1C rats, whereas the cross-sectional aortic area in the control groups remained unchanged by treatments (Figure 2A). Also, no significant differences were seen in wall area between 1K1C rats and controls treated with both losartan and tempol, which in combination can efficiently prevent arterial hypertrophy. PRA was shown to remain unchanged in 1K1C rats 2 weeks after surgery compared with control, uninephrectomized rats.27 Similar results are shown for the untreated 1K1C rats and controls in Figure 2B. Tempol treatment did not affect the PRA in either of the groups. In contradistinction, losartan treatment increased the PRA by 2.5-fold in both 1K1C and control groups (Figure 2B). When both losartan and tempol were added, PRA decreased by 38% compared with losartan alone but was still 1.8-fold higher than the tempol-treated and untreated groups. Also, no differences were seen between 1K1C rats and controls that received the same treatment (Figure 2B). Figure 3A shows ability of aortic rings from 1K1C and control rats with or without treatment to generate superoxide anions in vitro. Thoracic aortas from 1K1C rats generate twice as much superoxide anion in vitro compared with controls. Tempol or losartan treatment reduced superoxide generation by 30% and 23% respectively, compared with untreated 1K1C group after 2 weeks of treatment (Figure 3A). When tempol concentration was increased to 1 mmol·kg⁻¹·d⁻¹, a dosage level shown to efficiently reduce BP in spontaneously hypertensive rats,19 the superoxide generation by the aortic rings was not further decreased, which indicated a limited ability of in vivo tempol treatment to reduce
superoxide generation in vitro (data not shown). However, when losartan and tempol were added together, the in vitro ability of thoracic aorta from 1K1C rats to generate superoxide is decreased to the value of the treated controls. Also, the combined treatment has the ability to slightly though not significantly decrease superoxide generation in the control normotensive group (Figure 3A). To evaluate systemic oxidative stress in the different rat groups, plasma total isoprostanes were measured (Figure 3B). In this case, losartan and tempol alone or combined did not reduce plasma total isoprostanes in the 1K1C groups or control groups compared with untreated counterparts, which suggests that local oxidative stress probably is more relevant to reduction in BP.

Aortic nitrotyrosine was immunoassayed as an indirect measure of peroxynitrite formation in aortic wall. The results indicated an increased content of nitrotyrosine in 1K1C rats compared with controls in untreated groups as well as losartan- or tempol-treated groups (Figure 4). The increase ranged from 78% for the groups that received no treatment to 90% for the losartan-treated groups. In contrast, 1K1C rats treated with both losartan and tempol showed levels of nitrotyrosine similar to those of controls that received the same treatment, which indicate a strong effect of combined treatment on nitrotyrosine formation in the vascular wall (Figure 4). Finally, nitrotyrosine content did not significantly differ between control groups with different treatments. Figure 5 illustrates representative Western blots for nNOS protein expression in renal cortex and medulla of 1K1C and control rats with no treatment or treated with both losartan and tempol as well as group data from 4 rats per group. In the control groups (Figure 5B), the losartan plus tempol treatment did not significanly change the nNOS protein expression in both the cortex and medulla. On the other hand, in the 1K1C hypertensive rats with no treatment, a significant increase occurred in nNOS protein in cortex and medulla (Figure 5A) compared with untreated controls (Figure 5B), probably as a compensatory mechanism in the clipped kidney. Interestingly, in the 1K1C rats, losartan plus tempol treatment significantly decreased nNOS protein expression (Figure 5A) close to the detection limit in the cortex and proportionally in the medulla compared with both untreated 1K1C rats (Figure 5A) and normotensive, treated controls (Figure 5B).

**Discussion**

In the 1K1C rat model of renal hypertension, increased BP was shown to be independent of the renin-angiotensin system, at least starting with week 2 after surgery, as indicated by PRA levels.27 Also, remodeling of both large and small vessels in this model32,33 is similar to changes in vessel wall structure reported in human essential hypertension.34 The role of free radicals in BP regulation was reported for several models of experimental hypertension, including the spontaneously hypertensive4,5 and Ang II–perfused8 rat, but these models are dependent on the renin-angiotensin system to maintain high BP. Unlike these models, the 1K1C rat remains hypertensive even when AT1 receptors are blocked by losartan.35 Our results showed that 1K1C rats treated with losartan for 2 weeks have a moderate reduction in both BP and superoxide anion generation in vitro by the aortic rings, which suggests that Ang II is a significant contributor to both BP control and superoxide production. The apparent contra-
duction between these data and the data previously reported by us\textsuperscript{35} that showed that losartan cannot significantly reduce BP in 1K1C rats could be explained by the difference in the losartan dosage used in the 2 studies. In the present study, 50 mmol·kg\textsuperscript{-1}·d\textsuperscript{-1} of losartan administered represents twice as much as the dose used previously.\textsuperscript{35} Hence, at the present concentration, losartan might be more efficient for completely blocking AT\textsubscript{1} receptors. When the SOD mimetic concentration, losartan might be more efficient for completely blocking AT\textsubscript{1} receptors. When the SOD mimetic tempol was used in 1K1C rats for 2 weeks, both systolic and mean BP were moderately reduced, which indicates that superoxide anion is, at least in part, directly involved in BP regulation in this model. However, when both losartan and tempol were used, rats showed a strong decrease in BP and superoxide production, which suggests that the 2 parameters are well correlated. One may assume that when both the potential source of superoxide (ie, Ang II and the superoxide anion itself) are blocked, the effect is more potent than when the 2 processes are regulated individually. The synergistic effect of the 2 suggests that other indirect interactions are also involved in the regulation of BP. Also, superoxide anion was shown to have vasoconstrictor effects in vitro,\textsuperscript{13}\textsuperscript{34} but evidence concerning its long-term effect on vascular remodeling is missing. Interestingly, wall-area thickness, an indirect measure of arterial wall hypertrophy,\textsuperscript{35} perfectly parallels superoxide production in treated and nontreated animals. This phenomenon suggests a probable effect of superoxide on arterial hypertrophy, possibly through the action of the platelet-derived growth factor receptor, shown to be involved in hypertrophy in this model.\textsuperscript{12}\textsuperscript{38} A good index of oxidative stress is formation of 8-isoprostane, a stable product of lipid peroxidation.\textsuperscript{36}\textsuperscript{37} When we measured the total (free and esterified) levels of isoprostanes in plasma of 1K1C and control rats that received different treatments, the results did not show any significant differences between groups. A possible explanation is that only free or esterified isoprostane amounts have changed and that this is not necessarily reflected in the level of total circulating isoprostanes. Supporting this explanation is a recent study by Haas et al,\textsuperscript{38} in which Ang II increased free but not esterified or total 8-isoprostane levels in plasma of hypertensive pigs. Nitrotyrosine is considered a marker of peroxynitrite formation in vitro and in vivo.\textsuperscript{39} Peroxynitrite is produced by the reaction between the NO and superoxide anion.\textsuperscript{40} A drastic reduction in nitrotyrosine content of the thoracic aorta was detected only in the 1K1C rats treated with both losartan and tempol but not with the individual drugs. This paralleled reduced generation of superoxide by aortic rings and suggested a role of the latter in the formation of nitrotyrosine in these animals. To ascertain whether tempol and losartan treatment affected NO production in the kidney, we analyzed the mRNA level of nNOS in both kidney cortex and medulla. nNOS was shown to be the major NOS isoform responsible for regulation of renal blood flow in both cortex and medulla.\textsuperscript{41}\textsuperscript{48} In 1K1C rats, an increase in nNOS protein expression in both cortex and medulla is detected by Western blotting compared with that in control animals (Figures 5A and 5B). This might be an adaptive response in the clipped kidney in an attempt to restore in part decreased blood flow induced by renal artery constriction. However, when both tempol and losartan were added together, nNOS expression in both cortex and medulla was significantly reduced. Although hard to explain, this result may indicate that a reduction in nNOS expression could improve blood flow autoregulation in hypertensive rats, as reported in a recent report in a 2K1C model.\textsuperscript{44}\textsuperscript{45} The reduction in nNOS expression could also explain the reduction in PRA in rats treated with both losartan and tempol versus rats treated with losartan alone. Studies with knockout mice lacking the nNOS gene showed that the level of PRA is reduced as a result of the nNOS gene knockout, which suggests a direct role of kidney nNOS in renin release.\textsuperscript{46} Our study showed that oxidative stress is likely to play a role in BP regulation and arterial remodeling in hypertension in 1K1C rats. Blockade of AT\textsubscript{1} receptor combined with antioxidant treatment was able to reduce BP substantially in 1K1C rats. The reduction in BP was paralleled by a decrease in aortic wall hypertrophy, reduced superoxide generation, and reduced nitrotyrosine content of the thoracic aorta. Also, the treatment seems to have had a complex effect on the ability of the kidney to generate NO and renin. The synergistic effect of losartan and tempol suggests that additional, more-complex mechanisms that involve the interaction between free radicals and AT\textsubscript{1} receptors, possibly dependent on certain levels on free radicals locally produced in the kidney or arterial wall, are responsible for BP regulation in this model. Further studies will be necessary to unravel the exact mechanisms responsible for this interaction. Nevertheless, our results suggest that combined therapy using the AT\textsubscript{1} receptor blockade and antioxidants might increase the efficacy of treatment for some forms of hypertension in humans.

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