Oxidative Stress

Inhibition of nitric oxide synthase enhances superoxide activity in canine kidney

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Majid, Dewan S. A., Akira Nishiyama, Keith E. Jackson, and Alexander Castillo. Inhibition of nitric oxide synthase enhances superoxide activity in canine kidney. Am J Physiol Regul Integr Comp Physiol 287: R27–R32, 2004. First published March 25, 2004; 10.1152/ajpregu.00073.2004.—To evaluate the role of a potential interaction between superoxide anion (O2−) and nitric oxide (NO) in regulating kidney function, we examined the renal responses to intra-arterial infusion of a superoxide dismutase mimetic, tempol (0.5 mg·kg−1·min−1), in anesthetized dogs treated with or without NO synthase inhibitor, Nω-nitro-arginine (NLA; 50 μg·kg−1·min−1). In one group of dogs (n = 10), tempol infusion alone increased urinary excretion of 8-isoprostane (13.9 ± 3.6 pg·min−1) and antinatriuretic (67 ± 7), which returned to the control levels as reported in many in vitro and in vivo studies (4, 27, 28, 32, 34).

METHODS

The experiments were performed in accordance with the guidelines and practices established by the Tulane University Animal Care and Use Committee.

Experiments were conducted in mongrel dogs (11–21 kg body wt) of either gender. These dogs were given supplemental amounts of sodium chloride (1.5 g·kg body wt−1·day−1 for 3 days) added to the normal laboratory diet to achieve a positive sodium balance as reported in earlier studies in our laboratory (16, 17). On the day of the experiments, pentobarbital sodium was administered intravenously at 30 mg/kg body wt for induction of anesthesia and was supplemented throughout the experiment as needed. The level of anesthesia in animals was frequently checked by eliciting corneal reflex during the entire experimentation period to determine the need of supplemental doses of pentobarbital sodium. Auffed endotracheal tube was inserted and connected to an artificial ventilator set at a rate of 18 strokes/min with a stroke volume of 15 ml/kg body wt. Body temperature was maintained within normal range using an electric heating pad. Systemic arterial pressure (SAP) was monitored via a catheter.

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inserted into the right femoral artery and connected to a Statham pressure transducer (P23DC) and recorded on a polygraph (model 7D, Grass Instrument) as well as by the Biopac data-acquisition system. The left femoral artery was cannulated for collection of blood samples. The left jugular and the right femoral veins were cannulated for administration of insulin and isotonic saline, respectively. Isotonic saline was infused into the right femoral vein at a rate of 0.5 ml/min during the entire experimental period.

The left kidney was exposed retroperitoneally and devascularized by tying and cutting all the renal nerves projecting to the kidney from the aortorenal ganglion (15, 16, 17). Renal blood flow (RBF) was measured by placing an electromagnetic flow probe (Carolina Medical Electronics) around the renal artery, which was isolated from surrounding tissue. A curved 23-gauge needle cannula was inserted into the renal artery and connected to a pressure transducer for measurement of renal arterial pressure. Additional catheters were connected to the needle cannula for continuous infusion of heparinized saline (0.4 ml/min) as well as to prevent clotting in the cannula tip and for the intrarenal administration of drugs (tempol, NLA). Urine was collected from a catheter placed in the left ureter. After completion of the surgical procedures, a dose (1.6 ml/kg) of 2.5% solution of insulin in normal saline was administered into the jugular vein at least 45 min before the initiation of the experimental protocol followed by a continuous infusion of 0.03 ml/kg/min of normal saline as well as to prevent clotting in the cannula tip and for the duration of the experimental period. This dose of tempol was found effective in reducing urinary 8-isoprostane (a biological marker for endogenous O2 activity) excretion in these anesthetized dog preparations (see Fig. 5). It was also reported that similar doses of tempol caused reduction in 8-isoprostane excretion rate that was associated with an effective reduction in blood pressure in spontaneously hypertensive rats (27). Ten minutes after the initiation of the perfusion, two 10-min urine collections were made with corresponding arterial blood sample collections. Then NLA (50 μg·kg⁻¹·min⁻¹; Sigma Chemical) was added to the infusion line with tempol to examine the responses to combine reduction in NO and O2 activity (Fig. 3). All the data reported in RESULTS and shown in the figures are the average of the two clearance periods during each condition of the experimental protocol. Values are reported as means ± SE. Statistical comparisons of the differences in the responses within the group were conducted using one-way repeated-measures ANOVA followed by Newman-Keuls test. Comparisons of the responses between the groups were made using unpaired t-test. Differences in the mean values were deemed significant when probability values were ≤0.05.

RESULTS

During the control collection periods in the dogs used in the present study (n = 30), the mean values of plasma sodium, potassium, and hematocrit were 146.8 ± 1.2 meq/l, 3.5 ± 0.2 meq/l, and 45 ± 2%, respectively. There were no appreciable changes in these plasma parameters during the course of the experimental protocols.

Renal responses to tempol infusion before NLA administration. Infusion of tempol into the renal artery before NLA infusion (n = 10) did not cause any significant changes in renal parameters as illustrated in Figs. 1 and 2. There were no differences between the responses to tempol infused for 30 min (n = 5) and for 60 min (n = 5). Thus these results were pulled together and presented as a single response to tempol in the figures. It was observed that tempol infusion alone caused a slight but significant reduction in SAP (137 ± 3 to 133 ± 4 mmHg, P < 0.05). As illustrated in Figs. 1 and 2, there were no significant changes in RBF, renal vascular resistances (RVR), GFR, urine flow (V), sodium excretion (U_{Na} V), and fractional excretion of sodium (FE_{Na}). Tempol also did not cause significant changes in urinary excretion of potassium (U_{K} V; 0.5 ± 0.1 to 0.6 ± 0.1 μmol·min⁻¹·g⁻¹). After the addition of NLA to the intra-arterial infusion line with tempol for >30 min, there was an increase of 34 ± 5% (P < 0.05) in RVR and decreases of 24 ± 1% (P < 0.05) in RBF, 26 ± 8% (P < 0.05) in V, 45 ± 11% (P < 0.05) in U_{Na} V, and 36 ± 15% (0.05 > P > 0.1) in FE_{Na} (Figs. 1 and 2) without any significant change in GFR or U_{K} V. Addition of NLA to the
tempol infusion reversed the decrease in SAP (137 ± 7 mmHg).

Renal responses to tempol infusion in dogs pretreated with NLA. Intra-arterial infusion of NLA alone before tempol infusion (n = 12) resulted in an increase in SAP (141 ± 4 to 146 ± 4 mmHg; P < 0.05). Figures 3 and 4 illustrate the responses to NLA alone on renal hemodynamics and function. There was a decrease of 29 ± 4% (P < 0.05) in RBF and an increase of 48 ± 9% (P < 0.05) in RVR without changes in GFR. NLA infusion alone in these dogs also caused decreases of 47 ± 5% (P < 0.05) in V, 67 ± 4% (P < 0.05) in UNaV, and 61 ± 6% (P < 0.05) in FENa without a significant change in UNaV (0.5 ± 0.03 to 0.5 ± 0.04 μmol·min⁻¹·g⁻¹). These responses to NLA were similar to those reported earlier from our laboratory (16, 17).

Although tempol infusion before NLA administration did not cause appreciable changes in renal parameters (Figs. 1 and 2), administration of tempol to the NLA-pretreated dogs resulted in significant increases in V, UNaV, and FENa as illustrated in Fig. 4. However, there were no significant changes in RBF, RVR, or GFR (Fig. 3). During tempol infusion in NLA-pretreated dogs, SAP as well as UNaV also did not change significantly.

Comparison of the responses to NLA before and during tempol. There were some differences in the responses to NLA between the group in which NLA was given in the presence of tempol and the group in which NLA was first administered alone before tempol infusion. It was observed that the magnitudes of the renal excretory responses to NLA in tempol-pretreated dogs were significantly less (V, 26 ± 8 vs. 47 ± 5%, P < 0.05; UNaV, 45 ± 11 vs. 67 ± 4%, P < 0.05; FENa, 36 ± 15 vs. 61 ± 6%, 0.05 > P > 0.1) than the responses observed with administration of NLA alone. However, the differences in the magnitudes of the renal hemodynamic changes to NLA (RBF, 24 ± 1 vs. 29 ± 4%; RVR, 34 ± 5 vs. 48 ± 9%) were not statistically significant.

Responses to NLA and tempol infusion on urinary 8-isoprostane and nitrate/nitrite excretion. As mentioned earlier in METHODS, the urine samples from the seven dogs in which tempol was infused after NLA administration were analyzed for 8-isoprostane and nitrate/nitrite concentration. Figure 5 illustrates these responses in urinary excretion rate of 8-isoprostane (UISOV) and nitrate/nitrite (UNOxV). It was observed that intra-arterial infusion of NLA in these seven dogs resulted in increases in UISOV. After addition of tempol to NLA infusion, UISOV returned to the control levels. As expected, NO synthase inhibition by NLA administration caused decreases in urinary excretion rate of NO metabolites (UNOxV). During coadministration of tempol and NLA, UNOxV did not change significantly from the values obtained during NLA infusion alone.

Effects of 3-CP infusion on renal hemodynamics and function. In a separate group of dogs, infusion of 3-CP either before (n = 3) or during (n = 5) infusion of NLA did not cause any appreciable changes in renal parameters. The values of RBF, GFR, V, and UNaV (4.0 ± 0.1 ml·min⁻¹·g⁻¹, 0.82 ± 0.10 ml·min⁻¹·g⁻¹, 10.5 ± 3.3 μmol·min⁻¹·g⁻¹, and 2.3 ± 6 μmol·min⁻¹·g⁻¹, respectively) obtained during control periods were not significantly different during 3-CP infusion alone (4.1 ± 0.2 ml·min⁻¹·g⁻¹, 1.0 ± 0.2 ml·min⁻¹·g⁻¹, 9.5 ± 2.7 ml·min⁻¹·g⁻¹, and 2.0 ± 0.6 μmol·min⁻¹·g⁻¹, respectively). Similar to what was observed in the earlier group, intra-arterial infusion of NLA alone before 3-CP infusion in five dogs resulted in usually observed decreases in RBF (3.7 ± 0.2 to 2.8 ± 0.3 ml·min⁻¹·g⁻¹), V (7.6 ± 2.3 to 3.9 ± 1.6 μmol·min⁻¹·g⁻¹), and in UNaV (1.6 ± 0.5 to 0.8 ± 0.5 μmol·min⁻¹·g⁻¹) without significant changes in GFR (0.87 ± 0.17 to 0.75 ± 0.12 ml·min⁻¹·g⁻¹). However, in contrast to
the findings with tempol infusion, 3-CP infusion in these NLA-pretreated dogs did not cause any notable changes in \(\text{U}_\text{Na}, \text{V} \) (0.7 ± 0.4 \(\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\)), as well as in RBF (2.5 ± 0.2 \(\text{ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\)) and GFR (0.71 ± 0.12 \(\text{ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\)).

**DISCUSSION**

In the present study, it has been observed that intra-arterial administration of an SOD mimetic, tempol, causes diuretic and natriuretic responses in dogs pretreated with NLA but not in the intact condition before NO inhibition. Infusion of another nitroxide compound (3-CP) having similar chemical structure as tempol but with minimal SOD mimetic activity (9) did not cause such changes in renal excretory function in NLA-treated dogs, indicating that these responses to tempol were linked to its \(\text{O}_2^-\)-scavenging effect. The dose of tempol used in this study was seen as sufficient to normalize the enhanced \(8\)-isoprostane excretion rate (Fig. 5A) during NO blockade, indicating its maximal efficacy as an antioxidant. The dose for NLA was also used to achieve maximal inhibition of renal NO formation as in other studies conducted in our laboratory (16, 17). As \(8\)-isoprostane excretion is considered as a marker for endogenous \(\text{O}_2^-\) activity (19, 23, 24), this finding indicates that the reduction in NO level facilitates the increment in \(\text{O}_2^-\) activity in the kidney. Urinary excretion of NO metabolites, nitrates/nitrites (\(\text{U}_{\text{NOx}, \text{V}}\)), decreased during NLA administration as expected and remained unchanged during coadministration of NLA and tempol (Fig. 5B). As scavenging of \(\text{O}_2^-\) by tempol in NLA-pretreated dogs caused diuresis and natriuresis without changing \(\text{U}_{\text{NOx}, \text{V}}\), it is conceivable that such changes in excretory function were not related to NO but to the reduction in \(\text{O}_2^-\) level. It was also noted that pretreatment with tempol generally attenuated the excretory responses to NLA administration. These findings suggest that the renal excretory responses to NO synthase inhibition are at least partially influenced by the concomitant enhancement of endogenous \(\text{O}_2^-\) level.

There is a possibility that these effects of tempol may be linked to its action on renal sympathetic nerves (30) or antioxidant-induced changes in the release of norepinephrine from the nerve terminals (14). However, the present experiments were conducted in denervated kidneys to minimize any influence from concomitant alterations in renal sympathetic activity. The procedure for renal denervation employed in the present study was previously shown to completely abolish the changes in renal variables due to elicitation of cardiovascular reflexes that are mediated by alteration in renal sympathetic nerve (15). In the present study, we did not look at the possible changes in any humoral factor(s) in response to tempol administration. However, the involvement of such possible humoral factor(s) in the responses to tempol in the present study seems unlikely as we did not observe any appreciable changes in renal parameters during tempol infusion before NO inhibition. Further experiments may be needed to clarify this issue. It may also be argued that these renal responses to tempol may be attributed to the possible formation of \(\text{H}_2\text{O}_2\) due to the use of this SOD mimetic (4). However, such possibility is unlikely as it has been reported that the effect of \(\text{H}_2\text{O}_2\) is rather antidiuretic and antinatriuretic (4), an effect opposite to what has been observed in the present study.

The fact that there were no appreciable changes in renal parameters during tempol administration before NO inhibition (Figs. 1 and 2) indicates that the basal tissue concentration of \(\text{O}_2^-\) may be kept to a minimal level in the intact condition due to efficient antioxidant effect of endogenous NO (3, 8, 18), and thus there would be no \(\text{O}_2^-\)-scavenging effects of tempol. In other studies in rats (27, 28), it was also demonstrated that...
tempol administration had no significant effect on systemic blood pressure in Wistar-Kyoto rats but caused hypotension in spontaneously hypertensive rats. The results of the present investigation and from others (27, 28) indicate that the effects of scavenging \( \text{O}_2 \) depend on the existing endogenous level of \( \text{O}_2 \) production in the experimental animal. Although no supportive evidence is currently available, it can be speculated that the function of the enzymes responsible for endogenous production of \( \text{O}_2 \) may be limited by intact NO synthase activity, and these oxidative enzymes may become upregulated during NO synthase inhibition. Another recent study from our laboratory has also demonstrated functional evidence for the presence of enhanced NO bioactivity in mice lacking the gene for the gp91PHOX subunit of NAD(P)H oxidase (11). Thus it is reasonable to speculate that a disruption of NO synthase activity may be critically linked to an upregulation of NAD(P)H oxidase and/or other oxidative enzymes.

It was observed that tempol-induced increases in urine flow and sodium excretion in NO-inhibited dogs occurred in the absence of changes in RBF or GFR (Figs. 3 and 4). Previous studies have also shown that SOD inhibition (enhancement of \( \text{O}_2 \) level) in dogs could cause reductions in urine flow and sodium excretion without appreciable changes in RBF or GFR (16). Collectively, these data demonstrate that intrarenal \( \text{O}_2 \) can exert a direct influence on renal tubular reabsorptive function, leading to water and sodium retention. The exact mechanism by which \( \text{O}_2 \) can induce antiureasis and antinatriuresis is not yet clear. However, studies using isolated tubule preparation by Ortiz and Garvin (22) have shown that \( \text{O}_2 \) can stimulate NaCl reabsorption by the thick ascending limb through a mechanism that is linked to protein kinase C activation in the epithelial cells. The findings in the present investigation clearly demonstrated that endogenous \( \text{O}_2 \) exerts such direct action on tubular reabsorptive function primarily under conditions of reduced NO activity. These results are in agreement with our previous findings that the renal excretory responses to SOD inhibition are enhanced during NO synthase inhibition and provide further support to the renoprotective role of NO over the actions of endogenous \( \text{O}_2 \) (16).

8-Isoprostane is generally believed to be formed endogenously due to oxidation of membrane phospholipids by a potent oxidant, peroxynitrite, that is produced during in vivo chemical reaction between NO and \( \text{O}_2 \) (2, 10, 24). However, we observed an increase in urinary excretion of 8-isoprostane when endogenous NO production was inhibited by NLA administration (Fig. 5). This increase in 8-isoprostane excretion was ameliorated completely by the \( \text{O}_2 \) scavenger tempol, indicating that such increased formation of 8-isoprostane may be the result of direct peroxidation of lipids by \( \text{O}_2 \) (19, 23). Although tempol infusion normalized the enhanced excretion rate of 8-isoprostane and caused somewhat of a reversal of renal excretory responses to NO inhibition (Figs. 4 and 5), it did not cause reversal of the vasconstrictor response to NLA administration (Fig. 3). This finding may not be entirely unexpected as the improvement of endothelial dysfunction due to the scavenging of endogenous \( \text{O}_2 \) has been suggested to be linked with enhanced NO bioavailability (18, 35). In other words, the vasodilator effect of scavenging \( \text{O}_2 \) is primarily due to the action of NO in the vasculature. Thus tempol administration during effective blockade of NO synthase is not expected to cause a renal vasodilator effect. It may be argued that the applied dose of tempol might have been insufficient to cause a vasodilator effect. However, this possibility is unlikely as this dose of tempol was maximally effective in reducing the increased level of 8-isoprostane excretion to a near control value. The fact that tempol did not cause any vascular effect despite its effective blockade of enhanced \( \text{O}_2 \) activity during NLA administration suggests that NO inhibition exerted a dominant effect over the action of \( \text{O}_2 \) in mediating renal vasoconstriction.

In conclusion, the results of the present investigation indicate that under conditions of reduced NO activity there is an increase in endogenous \( \text{O}_2 \) level that influences renal excretory function. These data further support the hypothesis that endogenous NO provides a renoprotective effect against the actions of \( \text{O}_2 \) by acting as an antioxidative agent and thus suggest an interactive role for NO and \( \text{O}_2 \) in the physiological regulation of kidney function.

**Perspectives**

The findings in the present study and also from our previous study (16) indicate an important role for \( \text{O}_2 \) and its interaction with NO in the regulation of renal function. It has been shown that NO activity in many biological systems is enhanced by addition of the enzyme SOD, suggesting that interactions of \( \text{O}_2 \) and NO are a frequent occurrence in biological tissues (21, 25). Importance of \( \text{O}_2 \) and NO interaction in the regulation of many biological events has been increasingly appreciated due to the fact that under normal conditions, \( \text{O}_2 \) is a constant product of cellular metabolism (1, 6, 13). In fact, it was proposed in 1989 (31) that vascular endothelium had an intrinsic capacity to generate \( \text{O}_2 \) for regulatory purposes such as inactivation of NO. As a gaseous free radical, NO is highly reactive with other free radicals such as \( \text{O}_2 \) (10); thus it could be argued that the free radical reactivity of NO is in many ways an important physiological regulator of cellular function in vivo. It has been demonstrated that NO has an antiatherosclerotic effect by acting as a powerful inhibitor of membrane lipid peroxidation because of its ability to scavenge \( \text{O}_2 \) (3, 20, 26). Previous studies also provided evidence that NO reacts with oxygen free radicals and thus antagonizes the prooxidant properties of heme proteins in the biological tissues (8). An NO donor has also been shown to provide a cytoprotective effect in the ischemic tissue by reacting with excess oxygen free radicals in ischemia-reperfusion injuries (18). Thus it is conceivable that the interaction between NO and \( \text{O}_2 \) has a role in
maintaining a desired state of balance between oxidative and antioxidative processes in the kidney, any imbalance of which would lead to derangements in renal function.

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