Increased Renal Medullary H$_2$O$_2$ Leads to Hypertension

Ayako Makino, Meredith M. Skelton, Ai-Ping Zou, Allen W. Cowley, Jr

Abstract—We have recently reported that exaggerated oxidative stress in the renal medulla due to superoxide dismutase inhibition resulted in a reduction of renal medullary blood flow and sustained hypertension. The present study tested the hypothesis that selective scavenging of O$_2^-$ in the renal medulla would prevent hypertension associated with this exaggerated oxidative stress. An indwelling, aortic catheter was implanted in nonnephrectomized Sprague-Dawley rats for daily measurement of arterial blood pressure, and a renal medullary interstitial catheter was implanted for continuous delivery of the superoxide dismutase inhibitor diethyldithiocarbamic acid (DETC, 7.5 mg · kg$^{-1}$ · d$^{-1}$) and a chemical superoxide dismutase mimetic, 4-hydroxytetramethyl piperidine-1-oxyl (TEMPOL, 10 mg · kg$^{-1}$ · d$^{-1}$). Renal medullary interstitial infusion of TEMPOL completely blocked DETC-induced accumulation of O$_2^-$ in the renal medulla, as measured by the conversion rate of dihydroethidium to ethidium in the dialysate and by urinary excretion of 8-isoprostanones. However, TEMPOL infusion failed to prevent DETC-induced hypertension, unless catalase (5 mg · kg$^{-1}$ · d$^{-1}$) was coinfused. Direct infusion of H$_2$O$_2$ into the renal medulla resulted in increases of mean arterial pressure from 115 ± 2.5 to 131 ± 2.1 mm Hg, which was similar to that observed in rats receiving the medullary infusion of both TEMPOL and DETC. The results indicate that sufficient catalase activity in the renal medulla is a prerequisite for the antihypertensive action of TEMPOL and that accumulated H$_2$O$_2$ in the renal medulla associated with exaggerated oxidative stress might have a hypertensive consequence. (Hypertension. 2003;42:25-30.)

Key Words: oxidative stress ■ blood pressure ■ antioxidants ■ renal disease ■ sodium

A cute elevations of arterial blood pressure have been demonstrated to markedly increase arteriolar superoxide (O$_2^-$) production that might impair endothelial function and set the stage for increased reactivity to vasoconstrictor stimuli and hypertension.1,2 Recently, the participation of this vascular oxidative stress in the development of hypertension has been confirmed in different animal models. In angiotensin II (Ang II)–induced hypertension, it was found that NADH/NADPH oxidase, the enzyme that is primarily responsible for O$_2^-$ production under physiological conditions,3,4 was activated in the arterial wall. It has been proposed that increased O$_2^-$ production contributes to remodeling of the vascular wall and the increases in peripheral resistance that are associated with hypertension.1 In spontaneously hypertensive rats (SHR), O$_2^-$ production was found to be increased in different arterioles, and administration of superoxide dismutase (SOD) or a membrane-permeable, chemical SOD mimic, 4-hydroxytetramethyl piperidine-1-oxyl (TEMPOL), lowered arterial blood pressure in these hypertensive rats.4–6 Moreover, it has been reported that high salt intake significantly increases xanthine oxidase–mediated production of reactive oxygen species (ROS) in the arteries of Dahl salt-sensitive (Dahl S) rats but not in Dahl salt-resistant rats, and that treatment of Dahl S rats with ROS scavengers prevented salt-sensitive hypertension.7,8

In addition to the vascular action of oxidative stress in vascular remodeling, evidence is emerging that increased ROS in the kidney might also contribute importantly to the genesis and development of hypertension.8–12 In SHR, the antihypertensive effects of TEMPOL were shown to be associated with a selective increase in medullary blood flow (MBF) and a reduction of renal medullary vasoconstrictor effects of Ang II.10 In micropuncture studies, an increased production of ROS in the macula densa region of SHR has been attributed to a decrease in the bioavailability of locally formed nitric oxide, which might directly produce vasoconstriction or enhance tubular glomerular feedback response, resulting in increases in renal vascular resistance and hypertension.11 Moreover, the diuretic compound indapamide lowered arterial blood pressure and decreased renal oxidative stress or lipid peroxidation in parallel in Dahl S rats fed a high-salt diet,8 suggesting that reduction of ROS within the kidney might prevent hypertension. By determining the SOD activity and urinary F$_2$-isoprostanes, Meng et al13 demonstrated that hypertensive Dahl S rats exposed to 3 weeks of a high-salt diet exhibited significant reductions of renal SOD activity and elevations of F$_2$-isoprostanes, indicating a state of increased oxidative stress in the kidneys of these hypertensive rats. More recently, we have demonstrated that renal medullary interstitial infusion of the SOD inhibitor diethyldithiocarbamate (DETC) markedly reduced renal MBF and sodium...
excretion. Long-term medullary interstitial administration of DETC into the single remaining kidney of nonnephrectomized Sprague-Dawley rats produced sustained hypertension, with a concurrent decrease in renal MBF. These results indicate that exaggerated oxidative stress due to increases in O$_2^-$ in the renal medulla might be an important pathogenic mechanism resulting in hypertension.

On the basis of these observations, we hypothesized that selective scavenging of O$_2^-$ in the renal medulla would prevent the development of hypertension associated with exaggerated oxidative stress in this kidney region. To test this hypothesis, we examined the effects of long-term renal medullary infusion of TEMPOL in preventing sustained hypertension and enhanced renal medullary oxidative stress induced by chronic medullary inhibition of SOD. Because TEMPOL was found to be ineffective in preventing the development of hypertension even with normalized O$_2^-$ levels in this kidney region, the studies were then directed toward exploring the mechanism counteracting the antihypertensive effect of TEMPOL. By confusion of catalase into the renal medulla, we examined whether the production of hydrogen peroxide (H$_2$O$_2$) during infusion of TEMPOL counteracts its antihypertensive action. To simulate the hypertensive action of H$_2$O$_2$ derived from TEMPOL, we also examined the effects of long-term renal medullary infusion of H$_2$O$_2$ on arterial blood pressure and renal medullary interstitial H$_2$O$_2$ levels.

**Methods**

**Surgical Preparation for Long-Term Study and Hemodynamic Measurements**

Adult, male, uninephrectomized Sprague-Dawley rats (250 to 350 g; Harlan Sprague-Dawley, Madison, Wis) were used for all studies. Rats were maintained on water and standard rat chow (Purina) ad libitum. The rats were anesthetized with ketamine (100 mg/kg) and acepromazine (2 mg/kg) IM for surgical implantation of arterial and interstitial catheters described in detail previously. After 7 days of recovery from surgery, daily measurements of mean arterial pressure (MAP) were made from 9:00 AM to 12:00 noon with use of an online data collection (rate, 100 Hz) and analysis system described previously.

**Experimental Protocols**

Five groups of rats were surgically prepared for study as described here. Group 1: MAP was measured daily in rats receiving TEMPOL (10 mg·kg$^{-1}$·d$^{-1}$) by renal medullary interstitial infusion (r.i.) for 3 days, which was then combined with DETC (7.5 mg·kg$^{-1}$·d$^{-1}$; n=8) or saline (n=6) r.i. for 5 days. Group 2: Urine was collected for measurement of 8-isoprostanate in rats infused r.i. with DETC plus TEMPOL (n=6). Rats were then prepared for acute microdialysis studies for the determination of medullary interstitial [O$_2^-$]. Group 3: MAP was measured daily in rats receiving the same infusate combination as in Group 1 but with catalase (5 mg·kg$^{-1}$·d$^{-1}$ r.i.) added to the TEMPOL infusion (n=6 per treatment). Group 4: Rats were infused for 11 days with either TEMPOL alone (n=6) or TEMPOL plus catalase (n=5). DETC was not infused, because this SOD inhibitor interfered with the fluorescent quantification of H$_2$O$_2$ in the renal medullary interstitial fluid. At the end of the infusion period, each rat was anesthetized and prepared for acute microdialysis study, and H$_2$O$_2$ in the dialysate was quantified as described below. Group 5: MAP was measured daily in rats receiving H$_2$O$_2$ (1.5 to 2 mmol·kg$^{-1}$·d$^{-1}$ r.i.; n=7) for 5 days. At the end of the recording period on the fifth day, each rat was prepared for microdialysis study to measure interstitial H$_2$O$_2$.

**Determination of 8-Isoprostanate Excretion**

Twenty-four-hour urine samples were collected during the final day of DETC plus TEMPOL infusion. Samples were collected, extracted, and analyzed for 8-isoprostanate by enzyme immunoassay, as described in detail previously.

**Microdialysis Protocol and Biochemical Determinations of O$_2^-$ and H$_2$O$_2$**

On the last day of the long-term infusion experiments for animals in Groups 2, 4, and 5, rats were anesthetized with ketamine (30 mg/kg) and thiobutabarbital (40 mg/kg IP) and prepared for in vivo microdialysis of the left kidney, as we described earlier. In animals in which O$_2^-$ was measured, the microdialysis probe was equilibrated for 1.5 hours with a solution of 500 μmol/L dihydroethidium (DHE) and 1.25 mg/mL salmon DNA before beginning two 30-minute collections at a rate of 2 μL/min. The conversion of DHE to ethidium (Eth) was used as an index of O$_2^-$ production in the renal medullary interstitium, and measurements were made as we have previously described. Measured Eth fluorescence in the dialysate was compared with the fluorescence intensity of a standard curve to obtain the molar concentration of Eth, which represents the equivalent O$_2^-$ concentration.

In the H$_2$O$_2$-infused group of rats, the microdialysis probe was equilibrated for 1.5 hours before beginning two 30-minute collection periods for the measurement of H$_2$O$_2$ in the renal medullary interstitium. During the entire microdialysis period, the interstitial infusion of either TEMPOL, catalase, or H$_2$O$_2$ was continued at the same dose as used in the long-term phase of the study. A fluorescence spectrometric assay (Amplex Red hydrogen peroxide assay kit, Molecular Probes) was used to determine H$_2$O$_2$ levels in the renal interstitial fluid collected by dialysis. Amplex Red is a fluorogenic substrate with very low background fluorescence, which reacts with H$_2$O$_2$ with a 1:1 stoichiometry to produce highly fluorescent resorufin. Reactions of the dialysate and kit reagent were incubated for 30 minutes at room temperature in the dark, and then fluorescence intensity was measured by a microtiter plate reader at an excitation/emission wavelength of 560/590±10 nm. After background fluorescence was subtracted, [H$_2$O$_2$] of the renal interstitial dialysate was calculated by using a resorufin-H$_2$O$_2$ standard calibration curve generated with increasing concentrations of H$_2$O$_2$ (0 to 2000 nmol/L) and Amplex Red in the perfusate.

**Statistical Analysis**

Data are presented as mean±SEM. For statistical comparisons, 1-way ANOVA with repeated measures was used, and either the Dunn or Tukey multiple-range test was performed. All statistical analyses were performed on the raw data. P<0.05 was considered to be statistically significant.

**Results**

**Group 1**

**Effect of Renal Medullary Interstitial Infusion of TEMPOL on DETC-Induced Hypertension**

As summarized in Figure 1, infusion of TEMPOL into the renal medulla failed to prevent the increase in arterial blood pressure induced by DETC. MAP increased significantly, from a TEMPOL-infused control level of 118±2 to 138±4 mm Hg at the end of the first day of DETC infusion. MAP remained elevated at 128±2 mm Hg after 5 days of DETC infusion. The increase in MAP was sustained over the 3 days after termination of the DETC infusion (n=8). The pattern and amplitude of increase in arterial pressure were very similar to those induced by renal medullary infusion of DETC alone, as we reported previously. In rats receiving a medullary infusion of TEMPOL alone and saline, MAP...
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remained unchanged throughout the entire period of infusion (n=6).

Group 2

Effect of Renal Medullary Interstitial Infusion of TEMPOL on Urinary Excretion of 8-Isoprostanes and Medullary [O$_2^-$] in DETC-Infused Rats
In a previous study, we reported that renal medullary infusion of DETC significantly increased interstitial levels of O$_2^-$ to $411 \pm 135$ nmol/L Eth compared with $7.9 \pm 11$ nmol/L ethidium (Eth) following interstitial saline infusion. Likewise, urinary 8-isoprostane levels were significantly elevated from $4.1 \pm 3.5$ in saline-infused rats to $8.8 \pm 1.6$ ng/d in DETC-infused rats. To confirm the efficiency of TEMPOL to remove O$_2^-$, the present study measured O$_2^-$ levels in renal medullary interstitial dialysate in rats receiving a long-term medullary infusion of both TEMPOL and DETC. The DETC-induced increase in medullary O$_2^-$ levels was completely blocked by the coinfusion of TEMPOL in the renal medulla. In rats chronically infused with both TEMPOL and DETC into the renal medulla (r.i. DETC+TEMPOL), interstitial concentrations of O$_2^-$ were not different ($17.9 \pm 4.2$ nmol/L Eth) from the levels in saline-infused kidneys (n=6). Similarly, TEMPOL was also shown to prevent DETC-induced increase in urinary excretion of 8-isoprostanes measured as $4.2 \pm 0.9$ ng/d in rats receiving both TEMPOL and DETC (r.i. DETC+TEMPOL), a value not different from that previously measured in saline-infused animals.

Group 3

Effect of Renal Medullary Interstitial Infusion of TEMPOL Plus Catalase on DETC-Induced Hypertension
To address why TEMPOL failed to prevent DETC-induced hypertension, we explored the possibility that an increase of local H$_2$O$_2$ production during renal medullary infusion of TEMPOL was counteracting the action of this compound. TEMPOL is known to generate H$_2$O$_2$ in vitro during dismutation of O$_2^-$.[20] As shown in Figure 2, addition of catalase to the infusion of TEMPOL completely blocked DETC-induced hypertension; ie, there was no statistical difference in MAP between DETC- and saline-infused rats when catalase was coinfused with the TEMPOL into the renal medulla.

Group 4

Effect of Catalase on Renal Medullary Interstitial [H$_2$O$_2$] After Renal Medullary Interstitial Infusion of TEMPOL
To confirm the ability of TEMPOL to increase medullary levels of H$_2$O$_2$, and the ability of catalase to metabolize H$_2$O$_2$ produced from TEMPOL, microdialysis measurements were carried out in chronically infused rats. Renal medullary interstitial [H$_2$O$_2$] was increased after renal medullary infusion of TEMPOL alone but was not altered in rats with infusion of TEMPOL plus catalase. As summarized in the Table, [H$_2$O$_2$] was significantly increased in rats receiving an interstitial infusion of TEMPOL, from $190 \pm 18$ to $314 \pm 45$ nmol/L (n=5). In rats coinfused with TEMPOL and catalase, however, [H$_2$O$_2$] was not different from that in rats receiving saline infusion (n=5). It should be noted, however, that this H$_2$O$_2$ assay was performed in a group of rats without infusion of DETC, because DETC reacted with H$_2$O$_2$ indicators, resulting in assay errors. To address this problem, we tested >3 different H$_2$O$_2$ assay kits with fluorescent, chemiluminescent, or UV spectrometric detection methods. We also tried to separate H$_2$O$_2$ from DETC in our samples by using solid-phase columns or other methods. At the time of this report, we have not been able to measure H$_2$O$_2$ in DETC-containing samples. The results presented in the Table, however, provide strong, functional evidence that catalase can cleave H$_2$O$_2$ in renal medullary tissue.

Effect of Catalase on Renal Medullary Interstitial [H$_2$O$_2$] After Medullary Infusion of TEMPOL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dialysate H$_2$O$_2$, nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (r.i.; n=6)</td>
<td>190+18</td>
</tr>
<tr>
<td>TEMPOL (r.i.; n=6)</td>
<td>314+45*</td>
</tr>
<tr>
<td>TEMPOL+catalase (r.i.; n=5)</td>
<td>189+20</td>
</tr>
</tbody>
</table>

r.i. indicates renal medullary interstitial infusion.

*Significant difference (P<0.05) from the values obtained in saline-infused rats.
Concurrent scavenging of H$_2$O$_2$ was therefore necessary for a significant 3-fold elevation of renal medullary interstitial activity in various biologic systems ranging from molecular, membranes.21 This nitroxide compound has been reported to act as a genuine “SOD mimetic” producing antioxidative activity in various biologic systems ranging from molecular, cellular, and laboratory animal levels.21,22 Recent studies have shown that TEMPOL is capable of dismuting 2 O$_2^-$ molecules by a direct reaction with O$_2^-$ or its ·OOH form. In this dismutation reaction, however, TEMPOL produces H$_2$O$_2$ with a rate constant of 10$^7$ (mol/L)$^{-1}$ s$^{-1}$.21

Recently, administration of antioxidant enzymes such as SOD and catalase has been shown to prevent or treat hypertension.6,23 However, the potential benefits of systemic administration of SOD are limited because SOD does not permeate biologic membranes and is therefore unable to remove O$_2^-$ produced intracellularly.23 To overcome these limitations, the membrane-permeable and metal-independent TEMPOL has been utilized as an in vivo antioxidant for the removal of intracellular and extracellular O$_2^-$, and it has been reported to lower arterial blood pressure in several models of hypertension.8,23–25 In short-term experiments, we have demonstrated that renal medullary infusion of TEMPOL increased renal MBF and sodium excretion.17 Given the important role of MBF in the long-term control of arterial blood pressure, if TEMPOL induces chronic increases in MBF and sodium excretion, then this compound would be predicted to have an antihypertensive action. However, the present study demonstrated that long-term renal medullary interstitial infusion of TEMPOL alone failed to prevent the production and development of sustained hypertension associated with SOD inhibition. By biochemical analysis, it was found that although TEMPOL infusion reduced O$_2^-$ levels, it resulted in an accumulation of H$_2$O$_2$ in the renal medulla.

The accumulation in the renal medulla of H$_2$O$_2$ produced by TEMPOL might be related to the relatively lower levels of catalase in this region compared with other tissues, such as the renal cortex.26,27 This is consistent with our findings that by increasing catalase in the renal medulla, the antihypertensive effect of TEMPOL was unmasked. These results suggest that at least in this kidney region, high levels of catalase are a prerequisite for the antihypertensive action of TEMPOL or SOD. Taken together, the results suggest that exaggerated production of O$_2^-$ or H$_2$O$_2$ over the capacity of catalase-mediated cleavage might explain the hypertension observed in the present study.

There is a concern why TEMPOL effectively prevented hypertension when it was used in other hypertensive models.4–6 Although we have demonstrated that increased H$_2$O$_2$ production might be one of the important mechanisms countering the antihypertensive action of TEMPOL in the renal medulla with exaggerated oxidative stress, it remains unknown whether sufficient generation of H$_2$O$_2$ occurred in these earlier studies to override the antihypertensive effects of the SOD mimetic. There are 2 important differences between the present and previous studies that might account for the differences in results. First, TEMPOL in our study was administered directly into the renal medulla. This local delivery of TEMPOL produced high concentrations of H$_2$O$_2$ in the renal medulla, which, as the data indicated, would itself be hypertensive and counteract the antihypertensive actions of TEMPOL in this region of the kidney. In contrast, other investigators administered TEMPOL systemically, either orally or intravenously,4–6 so TEMPOL could thereby be converted into other effective components by the liver or other systems or TEMPOL-induced H$_2$O$_2$ could be metabolized systemically. The second reason for the differences...
between our results and others is that we administered TEMPOL into a region that was already under conditions of an exaggerated local oxidative stress induced by a powerful SOD inhibitor, DETC. Under this circumstance, large amounts of H$_2$O$_2$ could be produced from the reaction of TEMPOL with O$_2^-$. This is quite different from previous studies, in which TEMPOL was administered systemically to genetic models of hypertension, such as SHR or Dahl S rats, which have been shown to have less severe levels of renal oxidative stress than produced in the present study.

**Vasoconstrictor and Hypertensive Effect of H$_2$O$_2$ in the Renal Medulla**

Previous studies have shown that H$_2$O$_2$ can induce vasoconstriction in a number of arteries in vitro, such as rat aorta,\(^{28,29}\) rat pulmonary artery,\(^{30}\) canine basilar artery,\(^{31}\) and human placental arteries.\(^{32}\) Several mechanisms have been found to contribute to H$_2$O$_2$-induced vasoconstriction in these vessels, including an increase in Ca$^{2+}$ influx or Ca$^{2+}$ release from intracellular stores in smooth muscle cells; activation of protein phosphorylation enzymes such as phospholipase A$_2$, phospholipase C, protein kinase C, and tyrosine kinase; and stimulation of cyclooxygenase.\(^{1,28,32}\) Vasoconstrictor actions of H$_2$O$_2$ in the renal medulla would be expected to decrease renal MBF, reduce sodium excretion, and lead to hypertension.\(^{33}\) In a recent study, we demonstrated that short-term administration of H$_2$O$_2$ into the renal medulla significantly reduced renal MBF and sodium excretion.\(^{34}\) These results indicate that H$_2$O$_2$ is indeed a renal medullary vasoconstrictor and might be one of the important injury factors associated with oxidative injury in this kidney region under pathologic conditions. It should be noted that H$_2$O$_2$ has also been found to produce vasodilatation, especially in the coronary and cerebral circulation.\(^{35,36}\) The mechanism underlying observed differences in response to H$_2$O$_2$ in different vascular beds remains to be determined.

With respect to the role of H$_2$O$_2$ in the development of hypertension, there is accumulating evidence implicating this ROS in various forms of hypertension. In patients with essential hypertension, high plasma levels of H$_2$O$_2$ were reported.\(^{37}\) By using single-nucleotide polymorphism markers, a single-nucleotide polymorphism at 844 bp upstream from the start codon of the catalase gene was demonstrated to have a strong association with arterial blood pressure in patients with essential hypertension.\(^{38}\) In Dahl S hypertensive rats, plasma H$_2$O$_2$ levels were found increased,\(^{7,39}\) and the expression or activity of enzymes related to the metabolism of H$_2$O$_2$, such as catalase and glutathione peroxidase, was decreased in different tissues.\(^{1,34,40}\) In SHR, Ang II– or androgen-induced hypertension, and preeclampsia, H$_2$O$_2$ was also demonstrated to play an important role in mediating endothelial dysfunction, vascular hypertrophy, and enhanced vasoconstrictor responses.\(^{41–45}\) In the present study, we demonstrated that direct infusion of H$_2$O$_2$ into the renal medulla produced sustained hypertension, a response that could be blocked by catalase. These observations therefore provide direct evidence that H$_2$O$_2$ in the kidney, especially in the renal medulla, might serve as an important hypertensive factor. These results also suggest that antioxidant therapy that only enhances SOD expression and activity might not be effective in hypertension associated with exaggerated oxidative stress.

The present study did not attempt to explore the mechanism by which H$_2$O$_2$ induces renal medullary vasoconstriction and hypertension, especially the action mechanism of TEMPOL-induced H$_2$O$_2$ accumulation in the renal medulla. Because TEMPOL increased renal MBF but did not counteract the hypertension produced with SOD inhibition by DETC, it will be interesting to determine whether TEMPOL can still increase renal MBF or whether H$_2$O$_2$ exerts its hypertensive action through other mechanisms during SOD inhibition. Because H$_2$O$_2$ was found to decrease sodium excretion during renal medullary infusion, it is assumed that this ROS might result in sodium retention through its action on renal MBF and/or direct effect on tubular ion-transport activity and consequent blunting of the pressure-natriuresis response. In addition, the oxidative injury of renal medullary cells, such as endothelial cells, smooth muscle cells, or tubular cells, might also contribute to the development of hypertension associated with exaggerated oxidative stress. Further studies are needed to clarify these mechanisms.

In summary, the present study demonstrated that the chemical SOD mimetic TEMPOL failed to prevent DETC-induced hypertension unless catalase was infused into the renal medulla. Excess H$_2$O$_2$ accumulation in the renal medulla during TEMPOL infusion was found to counteract the antihypertensive action of this ROS. By direct infusion of H$_2$O$_2$ in the renal medulla, its hypertensive effect was further confirmed. It is concluded that excess accumulation of H$_2$O$_2$ in the renal medulla, either by dissmutation of O$_2^-$ or through other pathways, might produce hypertension.

**Acknowledgments**

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