Production and Actions of Superoxide in the Renal Medulla

Ai-Ping Zou, Ningjun Li, Allen W. Cowley, Jr

Abstract—The present study characterized the biochemical pathways responsible for superoxide (O$_2^-$) production in different regions of the rat kidney and determined the role of O$_2^-$ in the control of renal medullary blood flow (MBF) and renal function. By use of dihydroethidium/DNA fluorescence spectrometry with microtiter plates, the production of O$_2^-$ was monitored when tissue homogenate from different kidney regions was incubated with substrates for the major O$_2^-$-producing enzymes, such as NADH/NADPH oxidase, xanthine oxidase, and mitochondrial respiratory chain enzymes. The production of O$_2^-$ via NADH oxidase was greater ($P<0.05$) in the renal cortex and outer medulla (OM) than in the papilla. The mitochondrial enzyme activity for O$_2^-$ production was higher ($P<0.05$) in the OM than in the cortex and papilla. Compared with NADH oxidase and mitochondrial enzymes, xanthine oxidase and NADPH oxidase produced much less O$_2^-$ in the kidney under this condition. Overall, the renal OM exhibited the greatest enzyme activities for O$_2^-$ production. In anesthetized rats, renal medullary interstitial infusion of a superoxide dismutase inhibitor, diethyldithiocarbamate, markedly decreased renal MBF and sodium excretion. Diethyldithiocarbamate (5 mg/kg per minute by renal medullary interstitial infusion [RI]) reduced the renal medullary laser-Doppler flow signal from 0.6±0.04 to 0.4±0.03 V, a reduction of 33%, and both urine flow and sodium excretion decreased by 49%. In contrast, a membrane-permeable superoxide dismutase mimetic, 4-hydroxyetramethyl-piperidine-1-oxyl (TEMPOL, 30 μmol/kg per minute RI) increased MBF and sodium excretion by 34% and 69%, respectively. These effects of TEMPOL on renal MBF and sodium excretion were not altered by pretreatment with N$^G$-nitro-L-arginine methyl ester (10 μg/kg per minute RI). We conclude that (1) renal medullary O$_2^-$ is primarily produced in the renal OM; (2) both NADH oxidase and mitochondrial enzymes are responsible for the O$_2^-$ production in this kidney region; and (3) O$_2^-$ exerts a tonic regulatory action on renal MBF. (Hypertension. 2001;37[part 2]:547-553.)

Key Words: free radicals ■ oxygen ■ hemodynamics, renal ■ kidney

In contrast to the conventional idea that reactive oxygen species (ROS) are of only pathological consequence, recent studies have indicated that under physiological conditions, low concentrations of ROS play an important role in the normal regulation of cell and organ function.1-4 Redox-mediated signaling is emerging as a fundamental regulatory mechanism in cell biology and physiology.2,4 In this regard, ROS have been reported to participate in the control of vascular tone, and the interaction of superoxide (O$_2^-$) and NO has been considered as one of the important mechanisms regulating cardiovascular function.1,2,4,5 It has been demonstrated that O$_2^-$ inactivates the endothelium-dependent relaxing factor, thereby reducing the arteriolar dilation to acetylcholine or other endothelium-dependent vasodilators6 and that endothelial superoxide dismutase (SOD) activity significantly increased the half-life of the endothelium-dependent relaxing factor produced by acetylcholine.5 Recent studies have provided direct evidence that inactivation of SOD activity with diethyldithiocarbamate (DETC) selectively inhibits NO-induced vasorelaxation in coronary arteries.7 These results have indicated that the production or scavenging of O$_2^-$ may profoundly affect the level and activity of NO in the vascular wall. This interaction of NO and O$_2^-$ is of importance in the regulation of endothelial function and thereby the control of vascular tone.2,4

There is accumulating evidence indicating that oxidant stress critically contributes to the pathogenesis of hypertension and its related vascular disease.2,8-10 The first evidence that O$_2^-$ may play an important role in hypertension was provided by experiments demonstrating that an acute increase in arterial blood pressure markedly increased O$_2^-$ production in arterioles. Increase in O$_2^-$ may impair endothelial function and set a stage for increased reactivity to vasoconstrictor stimuli, resulting in the development of hypertension.2,3,4 Recent studies have demonstrated that increased ROS importantly participate in the development of hypertension in different animal models. In angiotensin II–induced hypertension, NADH/NADPH oxidase, which is primarily responsible for O$_2^-$ production under physiological conditions, was activated in the arterial wall.11-13 Increased O$_2^-$ production may contribute to remodeling of the vascular wall and an increase in peripheral resistance, resulting in hypertension.2,11-13 In

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spontaneously hypertensive rats, \( O_2^- \) production was found to be increased in different arterioles, and administration of SOD or a chemical SOD mimetic, 4-hydroxytetrathem-piperidine-1-oxyl (TEMPOL), lowered arterial pressure in these hypertensive rats.\(^1\)\(^8\)\(^11\)\(^14\) Moreover, high salt intake significantly increased xanthine oxidase (XO)-mediated ROS production in response to high salt intake in Dahl salt-sensitive rats but not in Dahl salt-resistant rats. Treatment of Dahl S rats with ROS scavengers prevented hypertension.\(^15\)\(^17\) Taken together, these findings indicate that ROS play an important role in the development of hypertension.

Despite a considerable amount of data indicating the role of ROS in the development of hypertension, the mechanisms by which ROS participate in the long-term control of arterial blood pressure remain to be clarified. Given the central role by which ROS participate in the long-term control of arterial blood pressure,\(^18\)\(^–\)\(^20\) we hypothesized that increased oxidant stress or an impaired antioxidant mechanism in the renal medulla may lead to reduction of renal MBF and sodium excretion and hypertension. The present study was designed to determine \( O_2^- \) production through different pathways in the renal medulla by fluorescence spectrometric analysis and to examine the role of \( O_2^- \) in the control of renal MBF and water/sodium excretion.

**Methods**

**Preparation of the Homogenate From Renal Tissues**

The dissected cortical, outer medullary, and papillary tissues were homogenized with a glass homogenizer in ice-cold HEPES buffer containing (mmol/L) sodium HEPES 25, EDTA 1, and phenylmethyl-sulfonyl fluoride 0.1. After centrifugation of the homogenate at 6000g for 5 minutes at 4°C, the supernatant containing membrane and cytosolic components, termed homogenate, was separated into aliquots, frozen in liquid N\(_2\), and stored at \(-80°C\) until use.

**Fluorescence Spectrometric Assay of \( O_2^- \) Production**

Fluorescence spectrometry of tissue \( O_2^- \) production was performed by using a modification of methods described by Benov et al\(^21\) and Mohazzab et al.\(^7\) Briefly, the fluorogenic oxidation of dihydroethidine to ethidium (Eth) was used as a measure of \( O_2^- \). Tissue homogenates (20 \( \mu \)g) freshly prepared from the different kidney regions were incubated with dihydroethidium (DHE, 10 \( \mu \)mol/L), salmon testes DNA (0.5 mg/mL) and the corresponding substrate for NADH/NADPH oxidase, mitochondrial respiratory enzymes, or XO in a microtiter plate at 37°C for 30 minutes, and Eth-DNA fluorescence was measured at an excitation of 475 nm and an emission of 610 nm by using a BJI-fluorescence microplate reader (Beckman).

NADH or NADPH oxidase activity to produce \( O_2^- \) was examined by the addition of NADH (0.1 mmol/L) or NADPH (0.1 mmol/L) as a substrate in the reaction mixture. Succinate (5 mmol/L) was used as a substrate for intramitochondrial \( O_2^- \) production, and antimycin (80 \( \mu \)mol/L) was used to block the normal reaction in the respiratory chain. It has been demonstrated that the incomplete hydrolysis of the substrates in the respiratory chain produces a large amount of \( O_2^- \).

Under normal conditions, respiratory chain enzymes use their substrates, such as NADH or succinate, to produce ATP rather than \( O_2^- \).\(^22\)\(^23\) Xanthine (0.1 mmol/L) was used as a substrate of XO. Salmon DNA (0.5 mg/mL) was added to bind to Eth and consequently stabilize Eth fluorescence, thereby increasing the sensitivity of \( O_2^- \) measurement (>40-fold).\(^7\)\(^22\)\(^23\) The enzyme activity of different pathways was prevented as fluorescence units per minute per milligram tissue homogenate.

**Animal Preparation for Renal Medullary Flowmetry**

Male Sprague-Dawley rats weighing between 250 and 300 g were fasted overnight but allowed free access to water. They were anesthetized with ketamine (30 mg/kg body wt IM) and thiobutabarbital (Inactin, 50 mg/kg body wt IP) and placed on a thermostatically controlled warming table to maintain body temperature at 37°C.

After tracheotomy, cannulas were placed in the right femoral vein and artery for intravenous infusions and measurements of arterial pressure. An abdominal incision was made, and the left kidney was placed in a stainless-steel cup to stabilize the organ for implantation of optical fibers to measure MBF and cortical blood flow (CBF) as previously described.\(^24\)\(^25\) For renal medullary interstitial infusion of drugs, a polyethylene catheter was implanted into the renal medulla. After implantations, a 0.9% NaCl solution was infused continuously at a rate of 0.6 mL/h to maintain the patency of interstitial infusion. The animals received an intravenous infusion of 2% BSA in a 0.9% sodium chloride solution at a rate of 1 mL/100 g per hour throughout the experiment to replace fluid losses and maintain a stable hematocrit of \(43±3\%\).\(^24\)

**Laser-Doppler Flowmetry of CBF and MBF**

Sprague-Dawley rats (250 to 300 g) were anesthetized and surgically prepared as described above. Laser-Doppler flowmeters (model PF3, PERIMED) were used to simultaneously determine the changes in CBF and MBF. Optical fibers were implanted, and laser-Doppler flow (LDF) signals were measured as we described previously.\(^25\)

Mean arterial pressure, CBF, and MBF were continuously monitored before and after renal medullary interstitial infusion of the SOD inhibitor DETC (0.5, 1, and 5 mg/min per kilogram body weight) or the SOD mimetic TEMPOL (30 \( \mu \)mol/min per kilogram body weight). Inasmuch as DETC has been reported to have a short half-life in vivo of \(5\) to 20 minutes,\(^26\) continuous renal medullary interstitial infusion was used to examine the effect of SOD inhibition on renal MBF and renal function. Doses of DETC chosen for the present study were the doses shown to produce 80% to 100% inhibition of SOD activity in previous studies.\(^27\)

The dose of TEMPOL used in our experiments has been reported to induce \(\text{N}^\text{6}\)-nitro-\(L\)-arginine methyl ester (L-NAME)–blockable reduction of arterial pressure.\(^14\) A single dose of TEMPOL was used because its action duration is relatively long, as has been reported previously.\(^14\) To determine the effect of NO synthase (NOS) inhibition on the response of MBF to TEMPOL, L-NAME (10 \( \mu \)kg per minute, a dose that blocked acetylcholine-induced increase in MFB)\(^28\) was infused into the renal medullary interstitial space for 1.5 hours, and then TEMPOL was infused. In these experiments, all compounds were infused into the renal medullary interstitial space to confine their effects in the renal medulla and limit systemic effects. Throughout the infusion of all these compounds, mean arterial pressure, CBF, and MBF were monitored.

**Effect of SOD Inhibitor or Mimetic on Renal Sodium and Water Excretion**

The rats were surgically prepared as described above. After surgery and a 1-hour equilibration period, urine was collected from both the left and right kidneys during two 20-minute control periods. DETC, TEMPOL, and L-NAME were infused as described above. Fifteen minutes after starting the infusion of these drugs, 2 or 3 twenty-minute urine samples were collected. Urine flow rate was determined gravimetrically. Sodium and potassium concentrations of urine and plasma samples were measured by use of a flame photometer. Urinary excretion data and renal blood flows were all factored per gram kidney weight.\(^25\)
Results

Determination of a Concentration-Dependent Eth Fluorescence and Superoxide-Induced Oxidation of DHE

As shown in Figure 1A, a linear relationship between fluorescence intensity and Eth concentrations was observed at a wave length of 475 nm for excitation and 610 nm for emission in the presence of salmon DNA. The minimal detectable concentration was 2 nmol/L. By use of xanthine and purified XO as an O$_2^-$-producing system, DHE was oxidized into Eth. Eth fluorescence intensity converted from DHE was xanthine/XO concentration dependent. However, the NO donor nonoate did not oxidize DHE into Eth; thus, there was no fluorescence detected when nonoate was added into the assay system even at a high concentration (1 mmol/L) (Figure 1B).

Production of O$_2^-$ Through Different Pathways in the Renal Cortex, OM, and IM

The results of these experiments are presented in Figure 2. The renal medullary infusion of the SOD inhibitor DETC (0.5 to 5 mg/kg per minute) produced a concentration-dependent decrease in renal MBF, but it had no effect on CBF (n=6). The renal medullary LDF signal was decreased from $0.6 \pm 0.04$ to $0.4 \pm 0.03$ V, a 33% reduction when DETC (5 mg/kg per minute) was infused into the renal medullary interstitium. During a 1-hour postcontrol period, MBF slowly returned but remained significantly lower than the control value at the end of 60 minutes. Renal medullary infusion of DETC had no effect on mean arterial pressure and CBF even at the highest dose (5 mg/kg per minute) examined in the present study.

Effect of Renal Medullary Interstitial Infusion of SOD Inhibitor (DETC) on Renal Water and Sodium Excretion

As depicted in Figure 3B, renal medullary interstitial infusion of DETC significantly reduced urine flow and sodium excretion (n=6). DETC at the highest dose studied (5 mg/kg per minute) reduced urine flow and sodium excretion by 49% and 48%, respectively. Potassium excretion was slightly decreased during renal medullary interstitial infusion of DETC at 5 mg/kg per minute.

Effect of Renal Medullary Interstitial Infusion of SOD Mimetic (TEMPOL) on CBF and MBF in Absence and Presence of L-NAME

Figure 4A presents the effects of renal medullary interstitial infusion of TEMPOL on CBF and MBF. TEMPOL produced a time-dependent increase in renal MBF, but it had no effect on CBF. During a 1-hour infusion, the renal medullary LDF signal increased to $0.82 \pm 0.04$ V from a control level of...
0.61±0.04 V, an increase of 34% (n=6). Renal medullary interstitial infusion of the NOS inhibitor L-NAME at a dose of 10 µg/kg per minute significantly decreased renal MBF with a 30% decrease in renal medullary LDF signal during 1.5 hours of infusion. In the presence of L-NAME, TEMPOL still increased the renal medullary LDF signal from 0.41±0.03 to 0.65±0.04 V (n=6). Under this condition, the renal cortical LDF signal was not significantly altered by TEMPOL.

**Effect of Renal Medullary Interstitial Infusion of SOD Mimetic (TEMPOL) on Water and Sodium Excretion in Absence and Presence of L-NAME**

The results of these experiments are presented in Figure 4B. Renal medullary interstitial infusion of TEMPOL for 60 minutes increased the urine flow from 16±0.8 to 29±2 µL/min per gram kidney weight and renal sodium excretion from 1.6±0.3 to 2.7±0.6 µmol/min per gram kidney weight, which represented an 81% increase in urine flow and a 69% increase in sodium excretion (n=6). TEMPOL was without effect on potassium excretion. In the presence of L-NAME in the renal medulla, TEMPOL still produced increases in urine flow and sodium excretion that were comparable to those observed in the absence of L-NAME.

**Discussion**

The present study detected O$_2^-$ production in renal cortical and medullary tissues in the presence of different substrates for O$_2^-$-producing enzymes by use of fluorescence spectrometry. This assay was based on the fluorogenic oxidation of DHE to Eth as a measure of O$_2^-$ and modified by the addition of salmon DNA in the assay mixture as a fluorescence...
enhancer. By use of a microtiter plate reader, this Eth-DNA fluorescence measurement can detect a minimal Eth concentration of 2 nmol/L. Before application of this assay to measure $O_2^-$ production in the renal tissues, a classic $O_2^-$-producing system, xanthine and purified XO, was used to determine the sensitivity and specificity of $O_2^-$-dependent fluorogenic conversion of DHE to Eth. After the addition of xanthine and XO into the assay mixture containing DHE and salmon DNA, an enzyme activity–dependent increase in Eth-DNA fluorescence intensity was observed. However, the addition of another well-known free radical, NO (nonoate), did not cause fluorogenic oxidation of DHE. These results suggest that the Eth-DNA fluorescence is dependent on the formation of $O_2^-$ in this assay mixture, which is suitable for the determination of enzyme activity for $O_2^-$ production.

There have been a number of approaches used in an effort to determine $O_2^-$ production in tissues or cells. In early studies, ferricytochrome c was used to quantify $O_2^-$ in vitro experiments, because it can be reduced by $O_2^-$. However, this protein was found to react not only with $O_2^-$ but also with other compounds with reducing activity. Moreover, it can serve as a substrate for cytochrome c reductase and cytochrome c oxidase. Therefore, the nonspecificity of this ferricytochrome c–based assay plagued its usefulness in the measurement of $O_2^-$. Electron paramagnetic resonance and mass spectrometry were also used for the measurement of $O_2^-$. These methods require expensive equipment and a large sample volume for assays. Many studies used lucigenin-enhanced chemiluminescence to estimate $O_2^-$ production in cultured cells, tissue homogenate, and intact vascular tissue. More recently, however, it was found that lucigenin can be auto-oxidized to produce $O_2^-$ during assay, which led to inaccurate and artifactual overestimation of the rates of tissue $O_2^-$ production. These properties of lucigenin limited its use in most tissues or cells with low rates of $O_2^-$ production.

Fluorescence spectrometric assay by oxidation of DHE has been extensively used for the measurement of intracellular $O_2^-$ concentration or enzyme activity for $O_2^-$ production in intact cells and vessels, because DHE easily enters into cells and produces fluorescence by the oxidation of $O_2^-$. In addition, Eth formed by oxidation of DHE can bind to DNA, which enhances fluorescence intensity by a factor of >40-fold. Therefore, this method is the most sensitive assay suitable for the measurement of intracellular $O_2^-$. Recently, this fluorescence assay of $O_2^-$ was extended to quantify $O_2^-$ concentrations in solutions. However, it was found that when a large amount of free $O_2^-$ or Eth is accumulated in the reaction mixtures, a chemical dismutation of $O_2^-$ can be activated. This may lead to an underestimation of $O^-$ concentration or production.

In the present study, salmon DNA was added into the assay mixtures to bind Eth, which increased the sensitivity of this assay and decreased the accumulation of free Eth in the assay mixture. We did not observe decreased Eth-DNA formation due to chemical dismutation even at 80 mU purified XO, an XO concentration that can produce 150 μmol/L $O_2^-$ by calculation, suggesting that this fluorescence assay can be used to accurately measure $O_2^-$, at least at this concentration range.

By use of this fluorescence spectrometry, $O_2^-$ production was detected in the renal cortical and medullary homogenates. It was found that the activity of NADH oxidase in the renal cortex was similar to that in the outer medulla and was much higher than that in the renal papilla. Mitochondrial respiratory chain enzymes to produce $O_2^-$ were mostly active in the outer medulla, among the 3 kidney regions. NADPH oxidase and XO expressed relatively less activity to produce $O_2^-$ in all kidney regions compared with NADH oxidase and mitochondrial enzyme–mediated $O_2^-$ production. These results suggest that under physiological conditions, $O_2^-$ in the kidney may be produced primarily by NADH oxidase and the mitochondrial enzyme system. In arterial tissues or cells, NADH oxidase has been indicated as a major enzyme responsible for $O_2^-$ production, which may contribute to endothelial dysfunction and remodeling of the vascular wall in hypertension. Our findings indicate that both NADH oxidase and mitochondrial enzymes may represent the major resource of $O_2^-$ production in the kidney. A recent study has demonstrated that XO activity can be detected in the rat kidney and increased in response to high salt intake in Dahl salt-sensitive rats. However, the activity of other enzymes responsible for $O_2^-$ production was not determined in those studies.

With respect to the regional distribution of NADH oxidase and the mitochondrial enzyme system for $O_2^-$ production in the kidney, we found that overall, the renal outer medulla exhibited a greater capability for producing $O_2^-$ than did the renal cortex and IM (papilla). Both NADH oxidase and the mitochondrial enzyme system exhibited great activity to produce $O_2^-$ in this kidney region. It is indicated that renal medullary $O_2^-$ may be derived primarily from NADH oxidase and mitochondrial enzyme systems. Inasmuch as both enzymes were found to produce more $O_2^-$ in response to tissue hypoxia, a low Po2 milieu in the renal medulla may activate these enzymes to produce a large amount of $O_2^-$, resulting in increased oxidant stress in this kidney region even under physiological conditions.

To determine the physiological significance of endogenously produced $O_2^-$ in the renal medulla, we examined the effect of renal medullary infusion of the SOD inhibitor DETC on renal MBF and water and sodium excretion. DETC markedly reduced renal MBF and urinary water and sodium excretion. These results indicate that endogenously produced $O_2^-$ in the renal medulla is largely scavenged by SOD and that $O_2^-$ produces vasodilation in this kidney region. In contrast, renal medullary interstitial infusion of a SOD mimetic, TEMPOL, significantly increased renal MBF and renal sodium excretion, further suggesting that $O_2^-$ is vasoconstrictive and antinatriuretic. Because a TEMPOL-induced increase in renal MBF was observed in the presence of an intact SOD system in the renal medulla, this TEMPOL-induced effect should be related to the scavenging of local free $O_2^-$. It seems that this free $O_2^-$ participates in the control of renal MBF and renal function. These results support the view that free $O_2^-$ is present and working in normal kidney tissues even with the ubiquity of scavenging systems.
There is a large body of evidence indicating that O$_2^-$ interacts with NO in the endothelium and thereby results in vasocostriction because of a decrease in NO-mediated vasodilation.$^{2,5,6}$ Recently, Schnackenberg et al.$^{14}$ reported that a SOD mimic, TEMPOL, significantly reduced arterial blood pressure in spontaneously hypertensive rats, and blockade of NOS by intravenous L-NAME abolished the TEMPOL-induced decrease in arterial blood pressure, indicating that the antihypertensive or vasodilatory effect of TEMPOL depends on its action to scavenge O$_2^-$ and unmask NO-induced vasodilation. When this interaction of NO and O$_2^-$ in the renal medulla is taken into consideration, it is possible that the TEMPOL-induced increase in renal MBF and sodium excretion was the consequence of increased NO concentrations resulting from the scavenging of O$_2^-$.

To test this hypothesis, we examined the effects of TEMPOL after the inhibition of NOS by renal medullary infusion of L-NAME. Unexpectedly, in the presence of L-NAME, TEMPOL still significantly increased renal MBF and sodium excretion, suggesting that the increase in NO due to the scavenging of a small amount of O$_2^-$ in the renal medulla does not contribute to TEMPOL-induced vasodilation. Other mechanisms may be involved in the effect of scavenging free O$_2^-$ by TEMPOL on renal MBF. It has been demonstrated that O$_2^-$ may directly increase intracellular calcium concentrations of vascular smooth muscle cells and thereby produce vasoconstriction.$^{1}$ Scavenging O$_2^-$ may reduce intracellular calcium concentrations and result in vasodilation. Moreover, O$_3^-$ has been demonstrated to inhibit the production of prostaglandin I$_2$.4,39 which is another potent vasodilator paracrine. In the renal medullary vessels, an increase in prostaglandin I$_2$ by scavenging O$_2^-$ may be an important mechanism, resulting in TEMPOL-induced vasodilation. More experiments are needed to further explore the mechanism by which O$_2^-$ produces vasoconstriction in the renal medullary circulation.

In summary, the present study has demonstrated that both NADH oxode and the mitochondrial enzyme system are primarily responsible for O$_2^-$ production in the renal medulla and that the renal OM is a major region for O$_2^-$ production in the renal medulla. Inhibition of SOD activity reduced renal MBF and water/sodium excretion, whereas scavenging of O$_2^-$ by TEMPOL increased renal MBF and water/sodium excretion. These results indicate that endogenously produced O$_2^-$ participates in the control of renal MBF and water/sodium excretion.

Acknowledgments

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