Increased Renal Medullary Oxidative Stress Produces Hypertension

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Abstract—The present study examined whether chronic increased oxidative stress within the medulla of the kidney lowers medullary blood flow and leads to hypertension. Optical fibers were implanted into the renal cortex and medulla of uninephrectomized Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, Wis) for the daily measurement of blood flow to these regions using laser-Doppler flowmetry techniques, while arterial pressure was measured from an indwelling aortic catheter. A renal medullary interstitial catheter was implanted for the continuous delivery of the superoxide dismutase (SOD) inhibitor, diethyldithiocarbamic acid (DETC), at a dose of 7.5 mg/kg/d. Renal interstitial superoxide (O$_2^-$) levels were determined by perfusing an O$_2^-$ sensitive fluorescent dye, dihydroethidium, through a microdialysis probe implanted into the medulla. Urine samples (24 hours) were collected for measurements of 8-isoprostane excretion. The results indicate that medullary DETC infusions increased tissue O$_2^-$ concentrations in the renal medulla (93.4±22.3, n=8, saline and 867.3±360.2, n=8, DETC; fluorescence units) and increased urinary 8-isoprostane excretion (4.1±0.4 ng/dl, n=9, saline and 8.8±1.6 ng/dl, n=10, DETC). Mean arterial pressure increased 24 hours after the start of intrarenal DETC infusion and remained nearly 20 mm Hg above control pressure throughout the 5 days of medullary SOD inhibition. During chronic medullary DETC infusion, medullary blood flow was significantly reduced (42.7%), whereas cortical blood flow was unchanged. Intravenous infusion of the same dose of DETC produced no changes in renal medullary or cortical blood flow or arterial blood pressure. The present experiments indicate that an increase in superoxide concentration within the renal medulla selectively reduces medullary blood flow resulting in chronic hypertension. (Hypertension. 2002;39[part 2]:667-672.)

Key Words: oxygen ■ hemodynamics ■ renal blood flow ■ blood pressure

Reactive oxygen species (ROS), such as superoxide anion radicals (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (·OH), and others, may play a critical role in the pathogenesis of hypertension and other pathological processes. It is well known that the ROS can result in cell damage by reacting with various cellular constituents, including membrane lipids, proteins, and DNA. There is also evidence that ROS can influence vascular reactivity either directly or through intermediate pathways such as reduction of nitric oxide (NO) availability or by oxidation of arachidonic acid with the generation of vasoactive lipid mediators.

It has been demonstrated that virtually all types of cells produce ROS. In addition to mitochondrial sources, ROS can be derived from nearly all oxidation reactions, including xanthine oxidase, cyclooxygenase, lipooxygenase, NO synthase, hemeoxygenases, peroxidases, hemoproteins such as heme and hematin, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Normally the levels of ROS are highly constrained in that there are many O$_2^-$ scavenging systems in cells and tissues that maintain tissue O$_2^-$ at a very low levels. One of the important pathways is superoxide dismutase (SOD), which produces the more stable ROS, H$_2$O$_2$, which in turn is converted to water by catalase and glutathione peroxidase.

There is evidence in the spontaneously hypertensive rat and Dahl salt-sensitive hypertensive rat that elevated production of ROS may contribute to the onset or progression of hypertension. Nakazono et al initially reported that a recombinant form of SOD, when injected intravenously, lowers blood pressure in spontaneously hypertensive rats. The effects, however, were short-lived. These studies were followed by other studies demonstrating that blood pressure could be lowered in several models of hypertension by treatment with liposome-encapsulated SOD or a stable, membrane-permeable, metal-independent SOD mimetic, Tempol. There is also indirect evidence that ROS may contribute to the development of human hypertension. Many studies have demonstrated an important role of ROS in the vascular pathologies related to atherosclerosis, diabetes, and heart disease.

Despite the well characterized role of the kidneys in both experimental and human essential hypertension, little is
known about the contribution of increased renal oxidative stress in the development of hypertension. It is unclear to what extent the antihypertensive responses to various antioxidant agents are associated with changes in renal function. We have found recently that the renal medulla is particularly sensitive to oxidant stress because the outer medulla is very rich in nicotinamide adenine dinucleotide (NADH) oxidase and mitochondrial enzymes that produce ROS. Studies in anesthetized rats also revealed that acute increases in medullary O$_2^-$ concentrations using the SOD inhibitor DETC produced significant reductions of medullary blood flow and that Tempol had opposite effects. Because a number of studies in our laboratory have demonstrated that chronic studies in our laboratory have demonstrated that chronic treatment with Tempol had opposite effects.

The present studies were designed to determine whether increased oxidative stress in the renal medulla would result in a reduction of medullary blood flow and lead to hypertension.

Materials and Methods

Surgical Preparation for Chronic Study

Adult, male uninephrectomized Sprague-Dawley rats (250 to 350 g) were used for all studies. The rats were anesthetized with ketamine (100 mg/kg) and acepromazine (2 mg/kg), and all surgeries were performed under aseptic conditions. Seven days after uninephrectomy, surgical implantation of arterial, venous, and interstitial catheters and optical fibers was performed as described previously. After surgery, rats received a continuous interstitial infusion of isotonic saline at a rate of 8 mL/min to maintain catheter patency and recovered for 7 days before study.

Hemodynamic Measurements

During the week after surgery, the rats were trained to rest for 2 hours each day in a tubular Plexiglas restrainer within their home cages. We then began to take daily measurements of mean arterial pressure (MAP), cortical blood flow (CBF), and medullary blood flow (MBF) using an on-line data collection (rate, 100 Hz) and analysis system as previously described. The flow signals from the renal cortex and medulla were measured and processed by a 2-channel laser-Doppler flowmeter (Transonics, Inc). Continuously recorded signals were transferred to minute averages for analysis.

Acute Measurement of O$_2^-$ Level in Renal Cortex and Medulla as Measured by Microdialysis

At the end of the experiments, the rats were anesthetized with ketamine (30 mg/kg IM) and Inactin (40 mg/kg; IP) and in vivo microdialysis of the kidney was performed as we have described it in a recent study. A microdialysis probe (Bioanalytical Systems) with a 0.5-mm tip diameter and a 30-kDa transmembrane diffusion cutoff was inserted into the renal medulla (5.5 mm in depth) from the dorsal surface and perfused with PBS (osmolarity 550 mOsm containing 500 μmol/L DHE and 1.25 mg/ml salmon DNA). In protocols in which DETC was delivered acutely into the renal medullary interstitium, a cortical microdialysis probe was inserted in addition to the medullary probe. The cortical probe was inserted to a depth of 2.5 mm and perfused with PBS (osmolarity 300 mOsm). In both protocols, the microdialysis probe was then perfused for 3 hours to enable the tissue to recover from the insertion of the probe and for equilibration to be established. During this time period the animal received an intravenous infusion of 2% bovine serum albumin in 0.9% NaCl at a rate of 1 mL/h per 100 g body wt. After the equilibration period, dialysate fluid was collected (50 μL; each volume) over two 25-minute intervals.

Conversion of DHE to Eth as an Index of Oxygen Free Radical Concentration

The dialysate was assayed to determine the conversion of dihydroethidium (DHE) to ethidium (Eth) as an index of O$_2^-$ concentration in the interstitium of the cortex and the medulla. For measurement of Eth in the dialysate, additional in vitro validation experiments were carried out to confirm the sensitivity of the Eth fluorescence curves across a wide range of concentrations. The standard curve was linear over a range from 0 to 250 nmol/L and the minimum detectable concentration (2×SD of background) was 15.6 nmol/L. We have performed in vitro experiments to examine the efficiency of dialysis for the superoxide anions using the DHE method. Twelve percent (±25%) of the superoxide anion in the interstitium was collected by this dialysis method as measured in vitro with xanthine/xanthine oxidase (X/XO; 5 μU/50 μM) as a superoxide producing system. DETC did not interfere with this reaction. We also conducted a series of experiments (data not shown) to determine the specificity of this assay. In those experiments we infused DETC interstitially in anesthetized rats to increase the Eth fluorescence signal. This increase in fluorescence was completely blocked by infusion of Tempol, a superoxide anion mimetic indicating this measurement is specific to superoxide.

The equilibration period (3 hours) prior to collection of the dialysis samples allowed enough time to establish a steady state concentration of superoxide and Eth/DNA complex in the medullary interstitium. Thus, any leakage of non-reacted DHE from the probe in this area would not be a concern.

Determination of 8-Isoprostane Excretion

Rats were housed in metabolic cages throughout the chronic studies and 24-hour urine samples were collected during the final day of DETC (or saline) infusion. Samples were collected into glass vials containing 50 μL of 0.5% butylated hydroxylamine to prevent oxidation during collection. The samples were kept cold during collection by packing the sample vials in insulated boxes filled with ice. The urine sample volumes were recorded, aliquoted, and frozen at −80°C for no more than 1 week before analysis.

Isoprostane was extracted from the urine samples before assay using an immunoaffinity column (cat number 416358; Cayman Chemical Corp). Aliquots of the urine (0.5 mL) were directly applied to the columns and the columns were washed with 2 mL of 0.1 mol/L PBS followed by 2 washes with 2 mL of water. The isoprostane was eluted from the column with 2 mL of a 95% solution of ethanol in water. Recovery of H-isoprostane added to the urine samples averaged 87% (n=13) with a coefficient of variation of 2.8%. The samples were then dried and reconstituted in EIA buffer and 8-isoprostane levels were measured using a commercial EIA kit (cat 516351; Cayman Chemical Corp). The standard curve ranged from 3 to 500 pg/mL. Overall recovery of 50 pg of 8-isoprostane added to 0.5 mL aliquots of urine averaged 81% without correction for the extraction losses (13%). Repeat analysis of a pool of rat urine yielded a mean value of 120±3 pg/mL (n=41 determinations) with an intraassay CV of 12.8% and an interassay CV of 15.3%.

Protocol 1: Effect of Chronic DETC Infusion into the Renal Medulla on the Urinary Excretion of 8-Isoprostane and the O$_2^-$ Levels in the Medulla

During the fifth day of medullary DETC infusion into the renal medulla at a dose of 7.5 mg/kg/d, a 24-hour urine sample was collected to determine 8-isoprostane excretion rates. After collecting this sample the rats were anesthetized and a microdialysis probe was implanted into the medulla of the left kidney. While continuing the medullary infusion of DETC or vehicle and after a 3 hour equilibration period, two 25-minute samples of dialysate were collected to determine Eth concentrations that represented O$_2^-$ levels in the dialysate.

Protocol 2: Effect of Chronic Infusion of DETC into the Renal Medullary Interstitium on MAP, CBF, and MBF

One week after recovery from surgical implantation of catheters and optical fibers, baseline measurements of MAP, CBF, and MBF were performed under anesthesia with ketamine (30 mg/kg IM) and Inactin (40 mg/kg; IP) and in vivo microdialysis of the kidney was performed as we have described it in a recent study. A microdialysis probe (Bioanalytical Systems) with a 0.5-mm tip diameter and a 30-kDa transmembrane diffusion cutoff was inserted into the renal medulla (5.5 mm in depth) from the dorsal surface and perfused with PBS (osmolarity 550 mOsm containing 500 μmol/L DHE and 1.25 mg/ml salmon DNA). In protocols in which DETC was delivered acutely into the renal medullary interstitium, a cortical microdialysis probe was inserted in addition to the medullary probe. The cortical probe was inserted to a depth of 2.5 mm and perfused with PBS (osmolarity 300 mOsm). In both protocols, the microdialysis probe was then perfused for 3 hours to enable the tissue to recover from the insertion of the probe and for equilibration to be established. During this time period the animal received an intravenous infusion of 2% bovine serum albumin in 0.9% NaCl at a rate of 1 mL/h per 100 g body wt. After the equilibration period, dialysate fluid was collected (50 μL; each volume) over two 25-minute intervals.
began. After at least 3 consecutive days of recording pressures and flows, the interstitial infusion was switched from isotonic saline to DETC (7.5 mg/kg/d) and continued for 5 days with measurements of MAP, CBF, and MBF determined on each day of the study. Saline was infused for 3 postcontrol days after cessation of the DETC infusion and measurements were made on each of these days.

**Protocol 3: Effect of Chronic Intravenous Infusion of DETC on MAP, CBF, and MBF**

One week after recovery from surgical implantation of catheters and optical fibers, 3 days of stable control MAP, CBF, and MBF measurements were obtained. An intravenous infusion of DETC was then begun at a dose of 7.5 mg/kg/d and continued for 5 days. After 5 days, DETC infusion was ended and saline was infused for 3 postcontrol days.

**Histological Analysis**

At the end of the experimental protocol the animals were killed and their kidneys were removed and then fixed in 10% formalin and paraffin embedded sections were prepared and stained. Positions of the interstitial catheter and optical fibers were also determined prior to histological sectioning. The PAS stained tissue sections obtained from three DETC-infused kidneys and three saline-infused kidneys were examined for evidence of tissue injury.

**Statistical Analysis**

Data are presented as mean±SEM. For statistical comparisons, 1-way ANOVA with repeated measures was used, and Duncan’s multiple range test as a post hoc test was carried out. All statistical analyses were performed on the raw data. *P*<0.05 were considered to be statistically significant.

**Results**

**Effect of Chronic DETC Infusion into the Renal Medulla on \( O_2^- \) Levels and 8-Isoprostane Urinary Excretion**

Figure 1 (top) demonstrates that after 5 days of intrarenal DETC infusion (7.5 mg/kg/d), \( O_2^- \) levels in the interstitial fluid of the inner medulla were markedly increased compared with saline infused control rats (saline, n=8; DETC, n=8). Consistent with this result, it was demonstrated that the urinary excretion of 8-isoprostane was significantly increased in the DETC infused rats (Figure 1, bottom; saline, n=9; DETC, n=10). An additional group of six rats was prepared for acute study with simultaneous microdialysis of both the cortical and medullary regions. These rats received a medullary interstitial infusion of DETC at the same dose infused chronically in the above group for 1 hour after a saline infused control period. In these animals, medullary Eth fluorescence increased 2.4 fold after DETC infusion while no change occurred in the cortex (data not shown).

**Effect of Chronic Infusion of DETC into the Renal Medullary Interstitium on MAP, CBF, and MBF**

The effects of chronic medullary infusion of DETC (7.5 mg/kg/d, r.i.) on arterial pressure and regional renal blood flows are summarized in Figure 2. Twenty-four hours after the start of the DETC infusion, MAP was significantly increased from an average control level of 121 mm Hg to 135 mm Hg. This increase was sustained at an average level of 139 mm Hg throughout the period of DETC infusion. The MBF fell by 23% on day 1 and by 43% on day 5 (top: saline, n=6; DETC, n=5) whereas CBF was unchanged (middle: saline, n=5; DETC, n=7). The lack of statistical significance in the fall of MBF on the first day of infusion is most likely...
because of the greater variability of the laser-Doppler flow measurement compared with the pressure measurement. MAP did not return to control during the 3 days after the end of the DETC infusion and on average tended to increase to higher levels. MBF began to return toward control levels on the third day of infusion. A group of rats were infused with saline alone into the medullary interstitium, and similar measurements made. There were no significant changes in MAP, MBF, or CBF when saline was infused for the same time course as DETC.

Effect of Chronic Intravenous Infusion of DETC on MAP, CBF, and MBF
Figure 3 shows that MAP, CBF, and MBF were unchanged by intravenous infusion of DETC (7.5 mg/kg/d). This was the same as the dose infused into the renal medulla and served as a control study to ascertain what the effects would have been had the DETC that was infused into the renal medulla escaped into the general circulation. The absence of any significant change of the measured hemodynamic variables indicates that even if all of the DETC that was infused into the renal medulla had recirculated, the systemic concentration that would have been achieved was not sufficient to produce hypertension or alter renal function.

Histological Review of Infused Kidneys
Microscopic examination did not reveal any abnormality of glomerular or tubular structures in either saline or DETC-infused kidneys. Furthermore, outer and inner medullary vasa recta appeared normal and there was no evidence of interstitial fibrosis.

Discussion
The results of these studies demonstrate that hypertension can result from increased levels of oxidative stress in the medulla of the kidney. Reduction of SOD activity in the renal medulla by the chronic infusion of DETC into the renal medullary interstitium of a single remaining kidney resulted in an increase of medullary superoxide anion concentrations, a reduction of blood flow to the renal medulla, and a sustained increase of arterial blood pressure. The data show that it was the increase of oxidative stress specifically within the renal medulla that was responsible for the development of the observed hypertension. This hypertensive effect of increased renal medullary $O_2^-$ was associated with a decrease in MBF. In a recent acute study in anesthetized rats, we reported that medullary infusion of DETC resulted in an immediate reduction of both medullary blood flow and sodium excretion within 30 minutes after start of infusion of this compound. Arterial pressure was unchanged during the acute 2 hour medullary infusion of DETC but significantly elevated 24 hours after the start of the chronic infusion. The present study found that MBF continued to be reduced after 24 hours of DETC administration and that arterial pressure was elevated by that time. Although electrolyte balance studies were not carried out, the slow onset of the hypertension suggests that the reduction of sodium and water excretion seen in the acute studies was sustained and resulted in a net positive sodium balance and volume expansion. Because no changes of cortical blood flow were observed in either the acute or present chronic medullary infusions of DETC, and because the reduction of medullary blood flow preceded the increase of blood pressure, these data indicate that hypertension was initiated through changes in renal medullary blood flow and subsequent sodium excretion. As recently reviewed, there is considerable evidence that preferential reductions of blood flow to the renal medulla of rats can initiate and sustain chronic hypertension.

The effective dose range to produce hypertension was found to be quite narrow in the present studies. In preliminary studies used to establish an effective hypertensive dose with minimum toxicity, we found that medullary delivery of 20 mg/kg/d resulted in initial hypertension for 2 to 3 days followed by a progressive and severe bradycardia and finally death from what appeared to heart failure. Yet, delivery of 5 mg/kg/d resulted in no chronic elevations of arterial pressure. The intermediate dose selected for the present studies (7.5 mg/kg/d) reliably produces a moderate form of hypertension with no apparent ill effects as evidenced by normal appetite, grooming, and activity of the rats.

The chronic intravenous delivery of DETC served as the control study to ascertain what the effects would be in the event that the DETC infused into the medullary interstitial space escaped into the systemic circulation. Because intravenous infusion of the same dose of DETC that was delivered into the renal medulla resulted in no measurable change of...
MAP, CBF, or MBF, these data provide further evidence that this novel model of hypertension was a direct consequence of increased oxidative stress within the renal medulla.

Evidence That Oxidative Stress Was Localized to the Renal Medulla

Studies were carried out to validate the assumption that medullary infusion of DETC actually increased tissue $\mathrm{O}_2^-$ concentrations within the kidney. This was clearly observed as shown by two different indices of oxidative stress in Figure 1 (increased urinary 8-isoprostane excretion and medullary $\mathrm{O}_2^-$ concentration as determined by microdialysis with DHE). The isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals.\textsuperscript{24} 8-Isoprostane is the major urinary metabolite of isoprostanes and is markedly elevated in the urine of rats after renal ischemia/reperfusion.\textsuperscript{7} The results of the DHE microdialysis studies confirm these observations and demonstrated that increased $\mathrm{O}_2^-$ levels occurred within the renal medulla in response to chronic medullary DETC administration.

Role of Oxidative Stress in Altering Renal Function and in Target Organ Damage With Hypertension

Increased $\mathrm{O}_2^-$ concentration could potentially modify renal medullary function in several ways. The $\mathrm{O}_2^-$ radicals could act directly on medullary vasa recta vessels to reduce blood flow which could then secondarily reduce sodium excretion. Recent work by Oritz et al shows that superoxide anion could stimulate NaCl absorption in the thick ascending limb.\textsuperscript{25} It has been reported that $\mathrm{O}_2^-$ may act directly to increase vascular smooth muscle tone by increasing intracellular Ca$^{++}$ in the vascular smooth muscle or altering other signaling mechanisms.\textsuperscript{2,26} $\mathrm{O}_2^-$ can also reduce NO availability to vascular smooth muscle and, thereby, raise vascular resistance.\textsuperscript{5,27} ROS could also act on tubular transport pathways in the deep medullary nephron segments and collecting ducts. $\mathrm{O}_2^-$ also oxidizes arachidonic acid within the cell membrane and can lead to generation of potent, vasoconstrictive metabolites such as isoprostanes.\textsuperscript{6,7} The increased urinary excretion of 8-isoprostane in the present study indeed suggests that products of lipid peroxidation could have participated in the reduction of medullary blood flow.

A number of studies have demonstrated enhanced levels of oxidative stress in vascular endothelial cells in experimental and genetic forms of hypertension.\textsuperscript{9,10,11,16,17,28} The specific mechanisms that predispose individuals with elevated blood pressure to development of target organ injury are not completely understood. It is interesting in the present study, despite the increased oxidative stress to the renal medulla in the DETC infused kidneys, that there was no evidence of interstitial nephritis, glomerular sclerosis, or alterations of the vasa recta vasculature. It seems that either longer exposure or more severe levels of oxidant stress or hypertension may be required for gross pathology of this nature to be observed. It does not appear, however, that such pathological changes could account for the failure of the arterial pressure to return to control levels during the first 3 days after medullary DETC infusion. This sustained elevation of pressure is interesting and with further study could provide clues related to the long-term effects of oxidant stress on renal function.

We conclude that elevation of $\mathrm{O}_2^-$ within the renal medulla can have an important influence on the long-term control of medullary blood flow, and a dysfunction of the normal scavenging systems in this medullary region of the kidney can lead to chronic hypertension.

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References

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