Superoxide dismutase and oxidative stress in Dahl salt-sensitive and -resistant rats

SHUMEI MENG, L. JACKSON ROBERTS II, GARRICK W. CASON, TRAVIS S. CURRY, AND R. DAVIS MANNING JR.
Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, Mississippi 39216; and Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232

Received 15 June 2001; accepted in final form 10 June 2002

In human hypertension, reactive oxygen species such as superoxide ions (O$_2^-$), hydroxyl radicals, and hydrogen peroxide (H$_2$O$_2$) contribute to the pathogenesis of hypertension and exacerbate renal damage. O$_2^-$ and H$_2$O$_2$ produced by leukocytes and plasma levels of lipid peroxides were increased in patients with uncontrolled hypertension (17). Blood pressure reduction in these patients decreased free radical production and lipid peroxides to normal (17).

Animal models of hypertension also have increased oxidative stress. The spontaneous hypertensive rat (SHR) is characterized by increased production of O$_2^-$ in mesenteric arterioles (22, 23). Recently, Schnackenberg and Wilcox (20) showed that oral administration of 4-hydroxyl-2,2,6,6-tetramethyl piperidine-1-oxyl (Tempol), a superoxide dismutase (SOD) mimetic, for 14 days reduced mean arterial pressure (MAP) in the SHR. In this study, Tempol significantly decreased urinary excretion of F$_2$-isoprostanes (20), which have been shown to be an index of oxidative stress in multiple pathological conditions (16). Oxidative stress may be increased in the Dahl salt-sensitive (S) rat, because vitamin E administration prevented renal and arterial injuries in S rats (1). However, the role of oxidative stress in Dahl salt-sensitive hypertension is not clear. In addition, the relationship between oxidative stress and renal damage in the S rat is not well understood.

Oxidative stress can be increased during hypertension by an increased production of reactive oxygen species such as O$_2^-$ or by a decrease in antioxidant enzymes such as SOD. The activity of SOD is decreased in patients with essential hypertension and experimental hypertension. SOD activity in erythrocytes (19) and neutrophils (4) is decreased in essential hypertension. Myocardial SOD activity is decreased in the SHR (5), and blood SOD activity is decreased in the Isiah stress-sensitive hypertensive rat (28). However, whether SOD is altered in S rats has not been determined.

Our goal was to test the hypothesis that renal oxidative stress is increased in the S rat during increased Na intake, and renal levels of SOD are decreased compared with the Dahl salt-resistant (R) rat. Studies were conducted in Dahl R and S/Rapp strain rats during either low or high Na intake for 1 to 3 wk. Oxidative stress was characterized by measuring O$_2^-$ production in the renal cortex and medulla and urine and plasma F$_2$-isoprostanes, and renal tissue Cu/Zn SOD and Mn SOD were determined to assess the level of this antioxidant enzyme in the kidney. Renal damage was assessed by measuring urinary protein excretion.

METHODS

Animal protocol. Experiments were conducted in 59 R and 61 S male rats, Rapp strain (Harlan, Indianapolis, IN) at an age of 7–8 wk. The project had the approval of the local...
Institutional Animal Committee. The rats were placed on either a low-Na (0.03%) or a high-Na (8%) diet for 1–3 wk. Rats were housed in a temperature-controlled room with a 12:12-h light-dark cycle.

The first group of R and S rats was subjected to the specified Na diet for 1, 2, or 3 wk, and a 24-h urine sample was collected for analysis of urine F$_2$-isoprostanes and protein. The Bradford (BioRad, Richmond, CA) method was used to measure urine protein concentration. The next day, rats were anesthetized with isoflurane, a laparotomy was performed, and blood was withdrawn from the aorta for analysis of plasma F$_2$-isoprostanes. All samples were stored at −80°C until processing. Urine and plasma F$_2$-isoprostane concentrations were measured with a gas chromatography, negative ion-chemical ionization mass spectrometry method (16).

Tissue preparation and Western blotting. After 3 wk on the specified Na diet, a second group of R and S rats was anesthetized with isoflurane, and kidneys were perfused through the aorta with 0.1 M phosphate-buffered saline containing 2% heparin (1,000 U/ml). The renal cortex and medulla were dissected out and were snap-frozen in liquid nitrogen buffer and stored at −80°C until processing. Renal cortical and medullary Cu/Zn SOD and Mn SOD protein were determined by Western blotting. There are three isoforms of SOD that localize in the cytosol (Cu/Zn SOD), the mitochondria (Mn SOD), or the extracellular space (ecSOD). Carlsson et al. (6) found that rat ecSOD is a dimer and has low affinity for heparin. Therefore, ecSOD content is very low in rat tissues (6), so the protein expression of this SOD was not measured in this study.

Frozen kidney sections were homogenized in 20 mM HEPES buffer (pH 7.5) containing protease inhibitors (100 μM pepstatin A, 1 mM phenanthroline, 100 μg/ml aprotinin, 100 μg/ml leupeptin, 1 mM PMSF, and 10 mM EDTA). Protein concentration was determined with the Lowry method. Equal amounts of protein from each sample were electrophoresed with a 4% polyacrylamide stacking gel and a 15% resolving gel. Separated proteins were transferred to nitrocellulose membranes and were incubated with the sheep anti-SOD antibody (Biodesign, Saco, ME). Membranes were washed and incubated with peroxidase-conjugated donkey anti-sheep/goat IgG (Biodesign). SOD protein was detected by chemiluminescence (ECL Plus kit, Amersham, Piscataway, NJ), quantified by densitometry (BioRad), and normalized relative to actin. Cross-gel comparisons were done using a common control.

Chemiluminescence measurement of O$_2^·$ production in renal tissues. Chemiluminescence of tissue in 5 μM lucigenin (bis-N-methylacridinium nitrate) was detected using a scintillation counter (Beckman LS 6500) in the out-of-coincidence mode with a single active photomultiplier tube (15). The validity of measuring oxygen free radical production with 5 μM lucigenin has been confirmed with electron spin resonance methods (11, 21). Renal cortical and medullary tissues were dissected out on dry ice, homogenized in lysis buffer (PBS containing: 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 10 U/ml aprotinin, 10 mM sodium orthovanadate), and the mixture was centrifuged. The supernatant was aspirated and kept on ice for free radical measurement. After 5 min of dark adaptation, 20 counts of 0.5 min each were made and only the last five counts were averaged and blank values were subtracted. The amounts of protein in the renal tissues were quantified with the Lowry assay. The final readings were expressed as counts per minute per milligram protein.

Data analysis. Statistics were performed by first using a two-way analysis of variance and a Fisher least-significant difference test for post hoc analysis at each experimental time point. Data were considered to be statistically different if $P < 0.05$. All data are expressed as means ± SE.

RESULTS

Urine and plasma F$_2$-isoprostane responses to a high- or low-sodium diet. Figure 1 indicates that urine F$_2$-isoprostane excretion, an index of oxidative stress in multiple pathological conditions (16), significantly increased throughout the experiment in S rats on a high-Na diet compared with R rats on a high-Na diet. Also, throughout the experiment, urinary F$_2$-isoprostane excretion significantly increased in R high Na-fed and S high Na-fed rats compared with low Na-fed rats of the same strain at the same experimental time. Except for week 1, urinary isoprostane excretion during low Na intake was not significantly different between R and S rats. At 3 wk, the urinary excretion of isoprostanes was 31.4 ± 7.4 ng/day in S high Na-fed rats compared with 22.0 ± 2.8 ng/day in R high Na-fed rats ($P < 0.05$). Urine concentrations of isoprostane (ng/ml) at 1, 2, and 3 wk were 0.36 ± 0.04, 0.31 ± 0.03, and 0.40 ± 0.05 for R high Na-fed rats; 0.86 ± 0.19, 0.89 ± 0.10, and 0.98 ± 0.14 for R low Na-fed rats; 0.36 ± 0.05, 0.36 ± 0.02, and 0.37 ± 0.35 for S high Na-fed rats; and
0.81 ± 0.24, 0.88 ± 0.23, and 0.89 ± 0.12 for S low Na-fed rats. Plasma F2-isoprostanes, an index of oxidative stress, are shown for R and S rats in Fig. 2. The average value for plasma F2-isoprostane concentration in the 2-wk S high Na-fed group was 0.65 ± 0.22 ng/ml, which was significantly greater than the value in the 2-wk R high Na-fed group (0.23 ± 0.03 ng/ml; \( P < 0.05 \)).

Renal cortical and medullary \( \mathrm{O}_2^- \) production responses to a high- or low-sodium diet. Figure 3A shows that renal cortical \( \mathrm{O}_2^- \) production significantly increased in S rats after 3 wk of high-Na intake, and the value was 68.7 ± 4.9 cpm/mg protein. Figure 3B shows that renal medullary \( \mathrm{O}_2^- \) production also significantly increased in S high Na-fed rats compared with R rats on either low- or high-Na intake. The highest renal medullary \( \mathrm{O}_2^- \) production occurred in S rats on high-Na intake, and the value was 37.3 ± 1.3 cpm/mg protein.

Renal cortical and medullary Cu/Zn SOD and Mn SOD responses to a high- or low-sodium diet. Figure 4 shows that renal cortical Cu/Zn SOD were not significantly different in the R and S high and low Na-fed groups in samples taken 3 wk after initiation of the Na diet. However, renal medullary Cu/Zn SOD was significantly lower in both the S high Na-fed and S low Na-fed groups compared with the R high Na-fed and R low Na-fed groups. Figure 5 indicates that in kidney samples taken 3 wk after initiation of the Na diet, the renal cortical Mn SOD was significantly lower in the S high Na-fed and S low Na-fed groups compared with the R high Na-fed group. Renal medullary Mn SOD was also significantly lower in both the S high Na-fed and S low Na-fed groups compared with the R high Na-fed group. The R high Na-fed group had a value of medullary Mn SOD of 22.7 ± 2.0 (densitometric units) compared with a value of 4.4 ± 1.1 ( \( P < 0.05 \) ) in the S high Na-fed group ( \( P < 0.05 \)). In addition, the S high Na-fed group had a lower value of medullary Mn SOD than the S low Na-fed group ( \( P < 0.05 \)).

Urinary protein excretion responses to a high- or low-sodium diet. Figure 6 shows that urinary protein excretion increased significantly at 1, 2, and 3 wk in S high Na-fed rats compared with the R high Na-fed group. Also, throughout the experiment, S high Na-fed rats also demonstrated a greater protein excretion than the S low Na-fed group. At 3 wk, the maximum value of urinary protein excretion in the S high Na-fed rats was 273 ± 32 mg/day, which was greater than the value of 35 ± 4 mg/day in the R high Na-fed rats at 3 wk ( \( P < 0.05 \)).

DISCUSSION

A major new finding in this study is that during a 3-wk increase in dietary Na in the S rat, renal oxi-
Changes in oxidative stress in S rats in the present experiment may have preceded major changes in arterial pressure and urinary protein excretion. Urinary isoprostane excretion in high Na-fed S rats reached a maximum value after 7 days of high Na, but arterial pressure of high Na-fed S rats in another study from our laboratory (14) reached significance for the first time on that day and had a value of 104 ± 4 mmHg. The maximum arterial pressure of 140 ± 3 mmHg was not reached until day 21 of high Na. After 7 days of high Na in S rats in the present experiment, urinary protein excretion was 94 ± 11 mg/day but increased to 273 ± 32 mg/day after 3 wk of high Na. Therefore, urinary isoprostane excretion reached a maximum after only 1 wk of high Na, and arterial pressure and urinary protein excretion continued to increase during the 3-wk period of high-Na intake. In another study from our laboratory, the increases in MAP and urinary protein excretion in S rats on high-Na intake for 3 wk were significantly blunted by long-term intravenous infusion of Tempol (14). This suggests that oxidative stress may contribute to the temporal increases in arterial pressure and renal damage in S rats on high-Na intake.

Increased oxidative stress can be caused by an increase in O$_2^\cdot_2$ production due to increased oxidase ac-

Fig. 5. Renal cortical (A) and medullary (B) Cu/Zn SOD expression in S and R rats on low- or high-sodium intake for 3 wk. Dens, densitometric. Insets show a representative Western blot of renal cortical and medullary copper/zinc (Cu/Zn) SOD, respectively. SOD protein shown in the insets corresponds to the groups immediately below in the bar graphs. †P < 0.05 compared with S low Na-fed rats in the same type of tissue. *P < 0.05 compared with S high Na-fed rats in the same type of tissue. N = 7 for each group.

Fig. 4. Renal cortical (A) and medullary (B) Cu/Zn SOD expression in S and R rats on low- or high-sodium intake for 3 wk. Dens, densitometric. Insets show a representative Western blot of renal cortical and medullary manganese (Mn) SOD, respectively. SOD protein shown in the insets corresponds to the groups immediately below in the bar graphs. †P < 0.05 compared with S low Na-fed rats in the same type of tissue. *P < 0.05 compared with S high Na-fed rats in the same type of tissue. N = 7 for each group.
have a deficient production or stability in renal medullary Mn SOD and Cu/Zn SOD and that salt-induced hypertension is associated with a further decrease in medullary Mn SOD protein expression.

Mn SOD is found in the mitochondria, and mitochondrial respiration is very high in renal tubules because of active transport mechanisms. This may increase leakage of \(O_2^-\), and because Mn SOD levels in the S high Na-fed rats are decreased in the renal medulla, \(O_2^-\) levels could increase, thus inactivating nitric oxide (NO) (12). Cu/Zn SOD levels are also decreased in the renal medulla of S rats, which could also increase \(O_2^-\) concentrations in the kidney. Thus these decreases in renal medullary SOD could lead to increased medullary \(O_2^-\) production, which could exacerbate the hypertension in S rats on high-Na intake.

Oxidative stress may have significant effects in human and experimental hypertension. Serum thiols and ascorbic acid were reduced in patients with hypertension, indicating an increased consumption of these antioxidants. Administration of vitamin C or other antioxidants decreased arterial pressure in these patients (7, 8) and improved the attenuated endothelial-dependent vasodilation in patients with essential hypertension (25). The SHR has high oxidative stress as indicated by elevated \(O_2^-\) production in mesenteric arterioles (23) and a reduction of arterial pressure in the SHR but not in the WKY rat using the SOD mimetic Tempol (20). The S rat may also have high oxidative stress as evidenced by increased \(O_2^-\) production in the mesentry and a high plasma \(H_2O_2\) concentration (24). Also, vitamin E administration improved renal and arterial injuries in S rats (1). These studies confirm our results in the present study that increased oxidative stress is associated with renal damage in the S rat.

Hayakawa and Raij (9) showed that in the S rat on a high-Na diet, there are parallel increases in urinary protein excretion, the glomerular injury score, and the tubulointerstitial injury score. Tubulointerstitial damage, which is highly correlated with the progression of renal disease, was more severe in the juxtamedullary and medullary regions (9). Therefore, although we only measured urinary protein excretion, which is due to oxidative stress, it is likely that medullary damage also occurred.

We measured arterial pressure in several groups of R and S rats, and the S rats but not R rats increase their MAP in a salt-sensitive fashion. Our studies showed that R rats remain normotensive on a high-Na diet (27) and S rats experience up to a 50-mmHg increase in MAP over 3 wk of a high-Na diet (14). During this period of time, the high Na-fed S rats also experienced an increase in glomerulosclerosis and glomerular cross-sectional area. These increases in MAP and glomerular damage in high Na-fed S rats were significantly blunted by long-term intravenous infusion of Tempol (14), an SOD mimic. This further supports our hypothesis that a lack of SOD in S rats contributes to renal damage and suggests that oxidative stress may contribute to the temporal increases in active transport mechanisms.

Fig. 6. Urinary protein excretion in S and R rats on low (A)- or high (B)-sodium intake for 1, 2, or 3 wk. \(tP < 0.05\) compared with R rats on the same Na intake at the same experimental time.
arterial pressure and renal damage in S rats on high-Na intake. Several recent studies suggest that high-Na intake in normotensive rats can increase oxidative stress. Sprague-Dawley (SD) rats were fed a low- or high-Na diet, and arteriolar and venular O$_2^\cdot$ production increased in the high Na-fed group (10). The O$_2^\cdot$ production decreased in high Na-fed rats treated with Tempol + catalase or SOD + catalase (10). These rats did not develop hypertension during high Na feeding, indicating that the increased O$_2^\cdot$ production was due to the high-Na diet but not hypertension. Other studies have shown that increased O$_2^\cdot$ could inactivate NO in SD rats on a high-Na intake, because the vasodilatory responses to NO agonists were markedly decreased in pial arterioles (13) and skeletal muscle resistance arterioles (12).

The above studies indicate that a high-Na diet even in normotensive rats can cause a release of O$_2^\cdot$ (2, 3, 10, 12, 13) and that a further increase in O$_2^\cdot$ may occur if a high-Na diet causes hypertension (12). In fact, this agrees with the present study, because a high-Na diet given to the highly salt-resistant R rat caused an increase in urinary F$_2$-isoprostane excretion. However, neither renal medullary nor cortical O$_2^\cdot$ production increased in the R rat on high-Na intake compared with low Na-fed R rats. This suggests that the increase in urinary isoprostanes may be caused by extrarenal elevations in oxidative stress. When the highly salt-sensitive S rat was given a high-Na diet in the present study, urinary F$_2$-isoprostane excretion increased even more than in the R rats. The decrease in renal SOD in the high Na-fed Dahl S rats could have contributed to this increased oxidative stress.

Release of O$_2^\cdot$ can have detrimental effects, including DNA destruction, protein aggregation, and lipid peroxidation, which can cause renal damage. F$_2$-isoprostanes are generated in a bound form when O$_2^\cdot$ react with arachidonic acid in phospholipids, and they are subsequently released in a free form that are excreted in the urine (18). F$_2$-isoprostanes have been used as a marker of oxidative stress in vivo (16). Elevated renal excretion of F$_2$-isoprostanes have been reported in renal ischemia-reperfusion injury (26) and during hypertension in the SHR (20). Treatment of the SHR with Tempol decreased the F$_2$-isoprostane excretion and arterial pressure significantly (20).

Urinary excretion of F$_2$-isoprostanes in the present experiment could have been increased in response to an increase in glomerular filtration rate (GFR) in the R and S high Na-fed rats. However, we previously showed that GFR is not different in high Na-fed R and S rats, and the increase in GFR in both groups compared with low Na-fed R and S rats is only 15% (27). Therefore, the increases in urinary F$_2$-isoprostane excretion in high Na-fed R and S rats are not likely due to an increase in GFR.

In conclusion, oxidative stress increased in S rats on high-Na intake as evidenced by increased renal cortical and medullary O$_2^\cdot$ production and increased plasma F$_2$-isoprostanes and urinary excretion of F$_2$-isoprostanes. After 3 wk on high Na, the S rats experienced an 81% reduction in Mn SOD levels and a 56% reduction in Cu/Zn SOD levels in the renal medulla compared with R rats. The Mn SOD levels of S rats were also decreased in the cortex. Therefore, the ability of SOD to inactivate O$_2^\cdot$ is likely decreased in the kidney. R rats on high-Na intake also experienced an increase in urinary F$_2$-isoprostane excretion but no increase in either renal cortical or medullary O$_2^\cdot$ production, which may reflect an increase in extrarenal oxidative stress. Compared with the R high Na-fed rats, the high Na-fed S rats showed an increase in renal O$_2^\cdot$ production, significantly greater increase in urinary F$_2$-isoprostane excretion, markedly lower renal SOD levels, and increased renal damage as evidenced by marked elevations in urinary protein excretion. A high-Na diet may increase renal oxidative stress in the S rat, which is accompanied by low renal SOD and is associated with severe renal damage.

This research was supported by Grant HL-51971 from the National Heart, Lung, and Blood Institute and National Institutes of Health Grants GM-42056, GM-15431, DK-26657, and CA-68485.

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AJP-Regul Integr Comp Physiol • VOL 283 • SEPTEMBER 2002 • www.aipregu.org


