NADPH Oxidase in the Renal Medulla Causes Oxidative Stress and Contributes to Salt-Sensitive Hypertension in Dahl S Rats

Norman E. Taylor, Padden Glocka, Mingyu Liang, Allen W. Cowley, Jr

Abstract—Dahl salt-sensitive (SS) rats exhibit increased renal medullary oxidative stress and blood pressure salt-sensitivity compared with consomic, salt-resistant SS-13BN rats, despite highly similar genetic backgrounds. The present study examined potential sources of renal medullary superoxide in prehypertensive SS rats fed a 0.4% NaCl diet by assessing activity and protein levels of superoxide producing and scavenging enzymes. Superoxide production was nearly doubled in SS rats compared with SS-13BN rats as determined by urinary 8-isoprostane excretion and renal medullary oxy-ethidium microdialysate levels. Medullary superoxide production in tissue homogenates was greater in SS rats, and the NADPH oxidase inhibitor diphenylene iodonium preferentially reduced SS levels to those found in SS-13BN rats. Dinitrophenol, a mitochondrial uncoupler, eliminated the remaining superoxide production in both strains, whereas inhibition of xanthine oxidase, NO synthase, and cyclooxygenase had no effect. 

Key Words: oxidative stress ▪ hypertension, renal ▪ renal disease ▪ sodium ▪ antioxidants
infused into the renal interstitium of rats fed a 4% NaCl diet to determine whether this enzyme is the source of increased $\text{O}_2^{-}$ production in SS rats.

Methods

Experimental Animals

Experiments were performed on inbred lines of male Dahl SS SS/JHsdMcwi (SS) and consomic SS-13BN rats maintained as inbred colonies at the Medical College of Wisconsin. The Medical College of Wisconsin Institutional Animal Care and Use Committee approved all of the experimental protocols. The rats were maintained ad libitum on tap water and a purified AIN-76A rodent diet containing 0.4% NaCl (Dyets) until it was changed to 4% in the chronic studies.

Acute Microdialysis for Renal Interstitial Nitrate/Nitrite and Superoxide Levels

SS and SS-13BN rats maintained on a 0.4% NaCl diet since weaning were placed in metabolic cages at 12 weeks of age, and 24-hour urine was collected for the determination of 8-isoprostane excretion as described previously. These rats were subsequently anesthetized with ketamine (30 mg/kg IM) and inactin (40 mg/kg IP), and in vivo microdialysis of the left kidney was performed as described previously. Baseline MAP measurements were recorded over 3 hours daily over 3 consecutive days using an online data collection and analysis system. In the second group, medullary dialysates were collected over two 30-minute intervals, and nitrite/nitrate concentrations were measured using a sensitive colorimetric method. The perfusing solution was then changed to include 500 μmol/L of dihydroethidium and 1.25 mg/mL of salmon DNA and allowed to equilibrate for 30 minutes followed by three 45-minute collection periods. 2-Hydroxyethidium (oxy-Eth) fluorescence was then measured at an excitation of 485 nm and an emission of 570 nm.

Tissue Collection and Preparation of Supernatant

Outler medullary (OM) tissue homogenates were prepared from a separate group of 12-week-old SS and SS-13BN rats fed a 0.4% NaCl diet since weaning for the measurement of enzyme activities and Western blots. Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg IP), the kidneys quickly removed, and the outer medulla separated from the cortex and the papilla before snap freezing on dry ice. The renal tissue was homogenized in a 10 mmol/L potassium phosphate buffer (pH 7.4) containing 250 mmol/L sucrose, 1 mmol/L EDTA, 2 mmol/L pepstatin, 1 mmol/L leupeptin, and 0.1 mmol/L phenylmethylsulfonyl. The homogenate was centrifuged at 10000 g for 5 minutes at 4°C, and the protein concentration of the supernatant was determined using a Coomassie blue protein assay with bovine serum albumin as a standard.

l-Arginine Quantification

OM tissue homogenates were deproteinized with 0.14 mol/L sulfoalicylic acid containing known concentrations of l-β-alanine (internal standard), centrifuged at 10000 g for 15 minutes, derivatized with o-phthalaldehyde (1 mg/mL), separated by reverse-phase high-performance liquid chromatography, and quantified by fluorometric detection as described previously.

NOS Activity

OM homogenate (250 μg) was incubated with appropriate cofactors, separated by isocratic reverse-phase high-performance liquid chromatography, and quantified by fluorometric detection as described previously.

Detection of $\text{O}_2^{-}$ Production by 2-Hydroxyethidium Fluorescence

OM homogenate (20 μg) was incubated with dihydroethidium (10 μmol/L), salmon testes DNA (0.5 mg/mL), and an inhibitor of NADPH oxidase (100 μmol/L diphenylene iodonium; DPI), xanthine oxidase (100 μmol/L oxypurinol), NOS (100 μmol/L l-NAME), cyclooxygenase (2 μmol/L meclofenamate), or dinitrophenol (250 μmol/L; DNP), an uncoupler of the mitochondrial respiratory chain, in a microtiter plate at 37°C for 35 minutes. The increase in oxy-Eth fluorescence was then measured at an excitation of 485 nm and an emission of 570 nm.

Total SOD, Catalase, and GPx Activities

Levels of these enzymes were determined by assay kits obtained from Cayman. Total SOD activity was assessed by the disappearance of superoxide detected by a tetrazolium salt. Catalase activity was determined as the conversion of methanol to formaldehyde in the presence of H$_2$O$_2$ using the method of Johansson and Borg. GPx activity was measured indirectly by measuring the decrease in NADPH absorbance at 340 nm, because it is converted to oxidized nicotinamide-adenine dinucleotide phosphate by glutathione reductase.

Western Blot Analysis

Expression of NADPH oxidase subunits (gp91phox, p22phox, and p47phox) was measured from OM homogenates by loading aliquots of 30 μg of protein into each lane to size separate by electrophoresis through a 10% SDS-PAGE gel. Proteins were then transferred onto a nitrocellulose membrane (Bio-Rad), blocked for 1 hour at 22°C, and incubated with primary antibody overnight at 4°C at a 1:200 dilution (gp91phox BD Biosciences; p22phox and p47phox Santa Cruz Biotechnology). Bound primary antibodies were detected with a horseradish peroxidase–labeled secondary antibody (1:200; 2 hours) and enhanced chemiluminescence (SuperSignal, Pierce Chemical). The band intensities were quantitated using densitometry (Scion Image, Scion) and normalized to β-actin.

Chronic Studies

Femoral arterial catheters were implanted into 2 groups of 9-week-old male SS and SS-13BN rats fed a 0.4% NaCl diet to measure unanaesthetized mean arterial pressure (MAP) as described previously. After a 6-day recovery, MAP was recorded in the first group for 3 hours daily over 3 consecutive days using an online data collection and analysis system. In the second group, medullary interstitial and femoral arterial catheters were implanted as described previously. Baseline MAP measurements were recorded over 3 consecutive days after a 6-day recovery. Renal interstitial apocynin infusion (12 mL/h) was then begun at a dose of 3.5 μg/kg per minute, a dose that effectively inhibited salt-induced $\text{O}_2^{-}$ production in the SS, using the minimum amount of ethanol needed to solubilize the apocynin (0.6% final concentration). Blood pressure was recorded for 3 days with the rats still maintained on a 0.4% salt diet. Control SS rats were continuously infused with either saline (8 μL/min, n=4) or ethanol vehicle (n=5). Because these 2 groups did not differ statistically from each other in any measured parameter, these results were pooled. All of the SS-13BN control rats were infused with vehicle. The rats were then switched to a 4.0% NaCl diet, and infusions continued for 7 more days with MAP recorded 3 hours daily. After the final pressure recording and collection of 24-hour urine for 8-isoprostane measurements, the rats were anesthetized and prepared for in vivo microdialysis measurement of renal interstitial nitrate/nitrite and oxy-Eth concentrations as described above.

Statistical Methods

Data are presented as mean±1 SEM. In cases where each animal served as its own control in the pretreatment and posttreatment periods, the data were analyzed using a 1-way ANOVA for repeated measures followed by a Tukey’s multiple range test. A P<0.05 was considered significant. Between-group comparisons were performed using a 2-way ANOVA followed by a Tukey’s multiple range test to compare individual time points.
Results

Increased Renal Medullary Oxidative Stress in SS Rats Fed a 0.4% NaCl Diet

SS rats fed 0.4% NaCl diet exhibited moderately (P<0.05) higher MAP (125±3 mm Hg, n=8) than SS-13BN rats (117±2 mm Hg, n=7) but substantially greater levels of renal oxidative stress as reflected by elevated urinary 8-isoprostane excretion (2415±173 versus 1468±266 pg/24 h). Direct evidence of increased oxidative stress within the renal medulla was obtained by determining oxy-Eth fluorescence in microdialysate of renal interstitial fluid of SS rats fed a 0.4% NaCl diet from weaning. SS rats exhibited 795±164 compared with 366±80 fluorescent units in SS-13BN rats (Figure 1). This increase was not because of differences in NO, as neither levels of NO (as determined from medullary interstitial nitrate/nitrite levels) nor the ability to produce NO, as reflected by OM tissue NOS activity and t-arginine concentrations, were significantly different between the SS and SS-13BN (Table 1).

Renal Medullary O$_2^-$ Production

Additional evidence of elevated O$_2^-$ levels in SS rats was found in renal OM tissue homogenates. Shown in Figure 2, 2-hydroxyethidium fluorescence of the SS control samples was significantly elevated above those of SS-13BN rats (9179±576 versus 5548±518 fluorescent units, n=6). To determine which enzymes were responsible for the increased O$_2^-$ production in SS rats, various inhibitors were administered to OM tissue homogenates. DPI, an inhibitor of NADPH oxidase, significantly decreased O$_2^-$ production in the SS samples to levels that were not significantly different than levels measured in SS-13BN (6861±449 versus 5752±404 U, n=6). No changes were seen by incubating with the xanthine oxidase inhibitor oxypurinol, nor with the NOS inhibitor L-NAME, nor the cyclooxygenase inhibitor meclofenamate, indicating that the pathways did not significantly contribute to renal medullary O$_2^-$ production in these strains when maintained on a 0.4% NaCl diet. In contrast, incubation with DNP, an uncoupler of oxidative phosphorylation, dramatically decreased O$_2^-$ production in SS samples (2444±354 versus control=9179±576 U, n=6) and virtually eliminated O$_2^-$ production in SS-13BN OM homogenates (222±287 versus control=5548±518 U, n=6), whereas the difference between the strains remained (2444±354 U SS versus 222±287 U SS-13BN). When DPI and DNP were combined, the remaining fluorescent signal from both strains was virtually abolished (511±306 U SS versus ~309±227 U SS-13BN) indicating that NADPH oxidase was responsible for this remaining O$_2^-$ production seen with DNP treatment alone in SS rats. These results indicate that the mitochondria are responsible for a basal production of O$_2^-$, which is quantitatively similar in both SS and SS-13BN rats, and that this production can be inhibited by uncoupling oxidative phosphorylation. Most importantly, these data show that NADPH oxidase is responsible for the elevated renal medullary O$_2^-$ levels in SS compared with SS-13BN rats, because DPI decreased O$_2^-$ production in OM homogenates of SS rats down to levels seen in the SS-13BN, whereas DPI had no effect in the SS-13BN.

Protein Expression of NADPH Oxidase Subunits

Western blots were performed on OM homogenates to determine the differential protein expression of the NADPH oxidase subunits gp91phox, p22phox, and p47phox. As shown in Figure 3, levels of gp91phox were substantially increased in SS rats compared with SS-13BN rats (659±73 versus 249±31 U, n=6). No differences were seen in p22phox or p47phox levels between the SS and SS-13BN strains.

### Table 1. Indices of NO in the Renal Outer Medulla

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>L-Arginine (nM/g tissue)</th>
<th>NOS Activity (pmol citrulline/min per microgram of protein)</th>
<th>Nitrate/Nitrite (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl SS</td>
<td>33.1±3</td>
<td>3.3±0.5</td>
<td>9.7±1.5</td>
</tr>
<tr>
<td>SS-13BN</td>
<td>27.6±3</td>
<td>3.6±0.8</td>
<td>11.0±2.4</td>
</tr>
</tbody>
</table>

*Significant from SS control, #significant from SS-13BN control, P<0.05. The 2 lines depicted serve as reference lines for the control values of SS rats (top line) and SS-13BN rats (bottom line).
Figure 3, protein levels of p22phox and p47phox were significantly greater in the outer medulla of SS rats (n = 6) compared with SS-13BN rats (n = 6), whereas levels of gp91phox were not different.

**Total SOD, Catalase, and GPx Activity**

To determine whether alterations in ROS scavenging also contributed to the elevated oxidative stress in the SS, total SOD, catalase, and GPx activity were determined in OM tissue homogenates of both strains (n = 6). No differences in activity were detected (Table 2), suggesting that these enzymes are not contributing to the differences in ROS between the strains.

**Effect of Renal Medullary Interstitial Infusion of Apocynin on Salt-Induced Hypertension and Medullary O$_2^-$ in SS Versus SS-13BN Rats**

Apocynin effectively reduced medullary interstitial O$_2^-$ levels in SS rats fed a 4% salt diet for 7 days, as seen in Figure 4, from 1059 ± 130 U in control rats (n = 9) to 422 ± 80 U in apocynin-infused rats (n = 6). These levels were comparable to those seen in vehicle-infused SS-13BN rats fed a 4% salt diet (437 ± 94 U, n = 6). No significant change in O$_2^-$ levels were seen in SS-13BN rats infused with apocynin (474 ± 77 U, n = 6). Parallel trends were seen in the urinary 8-isoprostane levels, but these changes were not of statistical significance. As shown in Figure 5, the effect on MAP of this preferential reduction of O$_2^-$ in SS rats was evident in that apocynin significantly reduced salt-induced hypertension in SS rats (157 ± 6 mm Hg apocynin versus 175 ± 4 mm Hg control), whereas MAP in SS-13BN rats was unaffected by apocynin infusion (127 ± 2 mm Hg apocynin versus 129 ± 4 mm Hg control). These data indicate that NADPH oxidase-dependent O$_2^-$ production significantly contributed to the development of salt-induced hypertension in the SS rat.

**Discussion**

Elevated oxidative stress in the renal medulla can lead to hypertension and initiate renal end organ damage. Therefore, the present study examined the pathways for the synthesis and degradation of O$_2^-$ to determine which enzyme(s) were responsible for increased oxidative stress in the renal medulla of SS rats. The results indicate that NADPH oxidase was the responsible enzyme, and selective reduction of renal medullary NADPH oxidase activity significantly reduced O$_2^-$ levels and greatly attenuated the salt-induced increase in arterial pressure in SS rats.

Although much has been done to demonstrate the contribution of ROS in the cortex on the development of hypertension, the renal medulla appears to be particularly vulnerable to oxidative stress, because it is the region of the kidney with the highest capacity to produce ROS but a diminished ability to scavenge them. We have demonstrated previously that O$_2^-$ production in the kidney of normotensive SD rats is predominately produced by NAD(P)H oxidase localized in thick ascending limb segments of the cortex and the outer medulla. Immunohistochemistry of the NADH oxidase subunits gp91phox has shown that the highest level of staining is in the outer medulla, with less staining in the cortex and no signal in the papilla. In contrast, the cortex appears to have a higher antioxidant capacity than the medulla, because SOD, catalase, and GPx activities were highest in the cortex of kidneys from Wistar rats.

A consequence of increased O$_2^-$ in the renal medulla is the development of blood pressure salt sensitivity by enhancing tubuloglomerular feedback and increasing tubular reabsorption in the medullary thick ascending limb. Additional increases in oxidative stress could then occur, because high pressure induces O$_2^-$ production in isolated

---

**TABLE 2. Activities of ROS Scavenging Enzymes in the Renal Outer Medulla**

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>SOD Activity (mM/mL/µg protein)</th>
<th>Catalase Activity (µmol formaldehyde/mL/min/µg protein)</th>
<th>GPx Activity (µmol NADPH/mL/min/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl S</td>
<td>58.0 ± 2.8</td>
<td>1.3 ± 0.2</td>
<td>3.52 ± 0.12</td>
</tr>
<tr>
<td>SS-13BN</td>
<td>57.5 ± 2.2</td>
<td>1.6 ± 0.2</td>
<td>3.70 ± 0.09</td>
</tr>
</tbody>
</table>
Consomic SS-13BN rats. The effects of systemic NADPH oxidase inhibition have been demonstrated previously in SD rats. The increase in $\text{O}_2^-$ production and elevation in blood pressure normally associated with aldosterone, deoxycorticosterone acetate-salt, and corticotropin-induced hypertension were reduced by administration of apocynin in the drinking water. Similarly, mice deficient in p47phox and infused with Ang II or deoxycorticosterone acetate salt showed no increase in vascular $\text{O}_2^-$ production and exhibited markedly blunted hypertension.

Although apocynin completely prevented the increase in $\text{O}_2^-$ in the renal medulla of the SS rat placed on 4% NaCl, it only partially attenuated the salt-induced hypertension. This is not surprising, because several physiological pathways contribute to the salt sensitivity of the SS including a deficiency in 20-hydroxyeicosatetraenoic acid and increased sympathetic tone. Interestingly, the same degree of protection from salt-induced hypertension was seen with renal interstitial apocynin infusion as was seen with medullary catalase infusion, suggesting that NADPH oxidase is the source of the oxidative stress but that $\text{H}_2\text{O}_2$ is the molecule exerting the physiological effect. At present, it is unknown what gene(s) on chromosome 13 protect the consomic strain from elevated physiological effect. At present, it is unknown what gene(s) on chromosome 13 protect the consomic strain from elevated

Another significant observation in the present study was that the activities of $\text{O}_2^-$ scavenging enzymes within the renal medulla were not different between the 2 strains fed a 0.4% sodium diet. Total SOD, catalase, and GPx activities were not significantly different, nor were there differences in indices of NO or the ability to produce NO before the onset of the high-salt diet and hypertension. This indicates that none of these pathways are of great significance in initiating the salt-induced increase in $\text{O}_2^-$ production in the SS rat or in buffering these responses.

We conclude that NADPH oxidase is the source of $\text{O}_2^-$ in the SS rat and that the resulting elevation in ROS contributes to salt-induced hypertension. Because SS rats share many of the abnormalities seen in human hypertension, administration of a specific NADPH oxidase inhibitor or a $\text{H}_2\text{O}_2$ scavenger...
may also be effective in treating SS hypertension in human patients.

Perspectives
The use of consomic animals for mapping important functional traits provides a powerful tool in guiding us toward genomic regions important in hypertension and other complex traits. Naturally, the phenotypic differences between the SS and SS-13BN provide a strong rationale to narrow and search for candidate genes of hypertension and/or renal disease on chromosome 13. As important, however, is the use of these model systems to understand the physiological pathways whereby these genes exert their pathophysiologic effect. The present study is just the beginning of this process and was conducted to help define the role that oxidative stress plays in the protection from salt sensitivity seen in the SS-13BN. Based on our current understanding of blood pressure regulation, there are a limited number of pathways that could be engaged to improve the sodium excretory function of the kidney and to protect the organism from salt-induced hypertension. It is likely that the gene or genes residing on chromosome 13 responsible for protection from salt-induced hypertension initiates a cascade of gene expression responses throughout the genome, evoking a set of known physiological mechanisms, which influence kidney function and blood pressure. Even if little is currently known of the causative gene(s), the present study represents how the overall functional pathways may have been modified by substitution of BN alleles from chromosome 13 in the genomic background of the SS rats.

Acknowledgments
This work was supported by the National Institutes of Health National Heart Lung and Blood Institute grants HL-29587, HL-54998, and HL-077263. Mr Taylor is supported by a predoctoral fellowship of the American Heart Association (AHA-04100437). We thank Meredith M. Skelton for careful review of the article and Brian Corson and Anne Ansley of the Biochemical Core Laboratory for the determination of NOS activity and 8-isoprostane levels. We appreciate the technical assistance of Jennifer Pietrusz and Kristi Usa.

References
NADPH Oxidase in the Renal Medulla Causes Oxidative Stress and Contributes to Salt-Sensitive Hypertension in Dahl S Rats
Norman E. Taylor, Padden Glocka, Mingyu Liang and Allen W. Cowley, Jr

_Hypertension._ 2006;47:692-698; originally published online February 27, 2006;
doi: 10.1161/01.HYP.0000203161.02046.8d
_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/47/4/692

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/