Abstract—NO synthase (NOS) can paradoxically contribute to the production of reactive oxygen species when L-arginine or the cofactor R-tetrahydrobiopterin (BH₄) becomes limited. The present study examined whether NOS contributes to superoxide production in kidneys of hypertensive Dahl salt-sensitive (SS) rats compared with an inbred consomic control strain (SS-13BN) and tested the hypothesis that elevated dihydrobiopterin (BH₂) levels are importantly involved in this process. This was assessed by determining the effects of L-nitroarginine methyl ester (L-NAME) inhibition of NOS on superoxide production and by comparing tissue concentrations of BH₁ and BH₂. A reverse-phase high-performance liquid chromatography method was applied for direct measurements of BH₁ and BH₂ using (S)-tetrahydrobiopterin as an internal standard. Superoxide concentrations were measured in vivo from medullary microdialysis fluid using dihydroethidine and in vitro using lucigenin. The results indicate the following: (1) that superoxide levels were elevated in the outer medulla of SS rats fed a 4% salt diet and could be inhibited by L-NAME. In contrast, L-NAME resulted in elevated superoxide production in consomic SS-13BN rats because of higher NOS activity; (2) SS rats showed a reduced ratio of BH₁/BH₂ in the outer medulla that was driven by increased concentrations of BH₂; and (3) lower superoxide dismutase and catalase activities contributed to elevated reactive oxygen species in SS samples. Based on the shift of BH₁ to BH₂ and the observation of L-NAME inhibitable superoxide production, we conclude that NOS uncoupling occurs in the renal medulla of hypertensive SS rats fed a high-salt diet. (Hypertension. 2006;48:1066-1071.)

Key Words: free radicals ■ hypertension ■ renal nitrergic oxide synthase ■ sodium

There is mounting evidence that NO synthase (NOS) can paradoxically contribute to the production of reactive oxygen species (ROS).¹ Normally, NOS catalyzes the conversion of L-arginine to NO and L-citrulline. However, when L-arginine or the cofactor tetrahydrobiopterin (BH₄) is limiting, both neuronal and endothelial NOS are capable of producing large quantities of superoxide (O₂⁻).²,³ This occurs because BH₄ normally aids in the stabilization of the NOS dimer and increases the affinity of NOS for L-arginine.⁴ When deficient, electron flow through the enzyme results in reduction of oxygen at the prosthetic heme site rather than formation of NO.⁵ BH₄ can be depleted via oxidation to 7,8-dihydro-L-biopterin (BH₂) when exposed to oxidant stress, thereby uncoupling NOS to its monomeric, O₂⁻-producing form.⁶ Uncoupled NOS is thought to be a prominent source of ROS in atherosclerosis,⁷ hypertension,⁸ cardiac hypertrophic dysfunction,⁹ and diabetic nephropathy.¹⁰

One commonly used model for the study of salt-sensitive hypertension is the Dahl S (SS) rat, because it exhibits many of the abnormalities that occur with hypertension in blacks.¹¹ In contrast, the genetically similar consomic SS-13BN rat is greatly protected from salt-induced hypertension, proteinuria, and glomerular disease.¹² It has been demonstrated that ROS are at least partly responsible for these pathologies,¹³-¹⁵ and whereas there is some evidence that uncoupled NOS contributes to O₂⁻ production in aortas of SS rats,¹⁶ it is unclear the extent to which this occurs in the kidney, particularly in the renal outer medulla, where renal interstitial fibrosis first occurs.¹⁷ Oxidative stress in this region of the kidney can profoundly influence arterial blood pressure as seen by the hypertension that develops in Sprague–Dawley rats when O₂⁻ or H₂O₂ levels in the renal medulla are elevated.¹⁸,¹⁹ Similarly, salt-sensitive hypertension was found to be reduced in SS rats when O₂⁻ or H₂O₂ was scavenged in this area of the kidney.

The present study examined whether NOS becomes uncoupled in the renal outer medulla of hypertensive SS rats as assessed in 2 ways: first, by determining whether O₂⁻ production could be inhibited by L-nitroarginine methyl ester (L-NAME) and, second, by determining differences in outer medullary tissue concentrations of BH₁ and BH₂. Recent in vitro data suggest that increased BH₂ with fixed levels of BH₁ can lead to O₂⁻ production by NOS and indicate that the ratio of BH₁/BH₂ determines uncoupling.²⁰ However, the method of Fukushima and Nixon,²¹ which is commonly used to quantitate biopterins, cannot measure BH₂. Therefore, a method was developed to accurately and sensitively quantitate various biopterins and biopterins from small quantities of biological samples.
tissue to determine whether elevated BH$_2$ could contribute to NOS uncoupling in vivo. In combination with the determination of l-NAME–inhibitable O$_2^-$ production, the results of these studies provide evidence of NOS uncoupling and increased renal medullary oxidative stress in the outer medulla of hypertensive SS rats compared with salt-resistant SS-13BN rats.

Methods

Experimental Animals
Experiments were performed using male Dahl salt-sensitive SS/JrHsdMcwi (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 (Dyets). The rats were maintained on 1 of 2 different diet regimens: (1) 0.4% salt diet: rats were maintained on a diet containing 0.4% NaCl from weaning or (2) high-salt diet: rats were fed the 0.4% NaCl diet until 6 weeks of age when the diet was changed to one containing 4% NaCl. All of the rats were studied at 11 to 12 weeks of age. For tissue harvest, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg IP), the kidneys quickly removed, and the outer medulla (OM) separated from the cortex and the papilla.

Acute Microdialysis for Renal Interstitial Nitrate/Nitrite and O$_2^-$ Levels
A group of male SS and SS-13BN rats were started on a 4% NaCl diet at 6 weeks of age. At 10 weeks, femoral arterial catheters were implanted as described previously. After a 6-day recovery, mean arterial pressure (MAP) was recorded 3 hours daily in unanesthetized rats within their home cages using an online data collection and analysis system. After 3 consecutive days of MAP measurements, the rats were subsequently anesthetized with ketamine (30 mg/kg IP), and the counts normalized per milligram of dry tissue weight.

Detection of O$_2^-$ Production by Lucigenin Chemiluminescence
O$_2^-$ production was estimated by lucigenin-enhanced chemiluminescence in separate groups of rats fed a 0.4% salt diet and a high-salt diet using a modification of the method of Munzel et al. The OM was separated and sectioned into 12 segments of ~2.5 mm. These segments were allowed to equilibrate in a Hanks’ balanced salt solution buffer containing 20 mM HEPES (pH 7.4) for 30 minutes and were then added to glass tubes containing 5 µmol/L of lucigenin in 2 mL of HEPES buffer or the buffer plus either 10 µmol/L of the flavo-enzyme inhibitor diphenyliodium (DPI) or 100 µmol/L of the NOS inhibitor l-NAME. These samples were then loaded in the luminometer (AutoLumat LB 953, Berthold Technologies) and dark equilibrated for 5 minutes. Each tube was counted in 5 consecutive 1-minute measurements and the data averaged and reported in relative light units per minute. After the assay, the tissues were removed from the buffer, dried at 50°C for 24 hours, and the counts normalized per milligram of dry tissue weight.

Enzyme Activity and Biopterin Tissue Collection and Preparation
Left kidneys were removed from 1 group of rats fed a 0.4% salt diet and another group fed a 4% salt diet for 5 to 6 weeks. The OM was separated and snap frozen on dry ice within 1 minute of removal. Tissues were homogenized in preweighed tubes containing 1 mL of 0.5 mol/L perchloric acid buffer with 2.7 mmol/L of EDTA, 12 mmol/L of l-ascorbic acid, 0.1 mmol/L of sodium thiosulfate; 50 ng of (6S)-5,6,7,8-tetrahydro-l-biopterin sulfate ([S-BH$_4$] Schirks Laboratories) was added as an internal standard, because rats do not produce this isomer. The homogenate was then centrifuged at 3000g for 5 minutes. The supernatant was removed and centrifuged again at 9000g for 15 minutes at 4°C. The supernatant was then snap frozen using liquid nitrogen and stored at ~80°C until analyzed, within 24 hours of collection.

Right kidneys were prepared for enzyme activity determinations as described previously by homogenizing OM tissue and centrifuging at 1000g for 5 minutes at 4°C. The protein concentration of the supernatant was determined using a Coomassie blue protein assay (Pierce) with BSA as a standard.

BH$_2$ and (6R)-5,6,7,8-Tetrahydro-l-Biopterin Dihydrochloride HPLC Assay
Endogenous amounts of (6R)-5,6,7,8-tetrahydro-l-biopterin dihydrochloride (R-BH$_2$) and BH$_2$ were measured using a variation of the reverse-phase (RP) high-performance liquid chromatography (HPLC) detection method originally described by Tani and Ohno. The supernatant was filtered in duplicate into HPLC vials using 0.45 µm of hydrophilic polytetrafluoroethylene filters (Millipore, SLCR-012-NL). The samples were separated by RP-HPLC using a 4.6×250-mm Spheri-10 5-µm column (K52915: Phenomenex). The samples were eluted using a linear gradient over 46.5 minutes with a mobile phase containing 0.1 mol/L of sodium phosphate buffer (pH 3.0) with 3 mmol/L of sodium octylsulfate (Acros Organics), 0.1 mmol/L of disodium EDTA, 0.1 mmol/L of ascorbic acid, and 5% (vol/vol) methanol as a gradient elution at a rate of 0.5 mL/min. The effluent from the column underwent postcolumn derivatization to oxidize BH$_3$, R-BH$_4$, and S-BH$_4$ into pterins, which are highly fluorescent. This was done by mixing the effluent from the HPLC column with 5 mmol/L of sodium nitrite solution (pH 3.0 adjusted with H$_2$SO$_4$) at a rate of 0.5 mL/min and heating the mixture to 80°C in a postcolumn reactor (model 310; Supelco). It is important to emphasize that H$_2$SO$_4$ must be used to adjust pH, because other acids interfere with the assay. The reaction was then cooled before fluorescence detection (model 474; Waters Instruments) using an excitation of 350 nm and an emission wavelength of 440 nm. The amount of BH$_3$, R-BH$_4$, and S-BH$_4$ in the sample was determined by comparing the area of these peaks with that of the internal standard (S-BH$_4$, 50 ng) and then normalizing the values per milligram of tissue.

NOS, Superoxide Dismutase, Catalase, and Glutathione Peroxidase Activities
A total of 250 µg of OM homogenate was incubated with appropriate cofactors, separated by isocratic RP-HPLC, and NOS activity determined from the ratio of the total l-[3H]arginine converted to l-[3H]citrulline and the amount of total arginine in the reaction as we have described previously. A total of 0.4 mmol/L of L-proline was added to the cofactor mix to inhibit arginase activity. Superoxide dismutase (SOD), catalase, and glutathione peroxidase activities were determined by assay kits obtained from Cayman as we have described previously.

Statistical Methods
Data are presented as mean±1 SEM. A P<0.05 was considered significant. Between-group comparisons were performed using a paired t test or 2-way ANOVA, as appropriate, followed by a Tukey’s multiple range test to compare individual time points.

Results

Renal Medullary O$_2^-$, Nitrate/Nitrite, and NOS Activity
SS rats fed a 4% NaCl diet for 6 weeks were significantly hypertensive, with a MAP of 184±7 mm Hg (n=8) compared with 138±3 mm Hg in SS-13BN rats (n=8) (Figure 1). In addition, O$_2^-$ production within the renal medulla of SS rats was nearly doubled, as determined by oxy-ethidium fluorescence in microdialysate of renal interstitial fluid. Renal
salt diet, whereas having no effect in SS-13BN rats, suggesting similar levels in the OM of SS rats fed either a 0.4% or a 4% diet, as shown in Figure 2. When dietary salt increased, $O_2^-$ production in either strain when fed a 0.4% salt diet, as shown in Figure 2. When dietary salt increased, $O_2^-$ production in either strain when fed a 0.4% salt diet, was significantly lower than our previously reported values in SS and SS-13BN rats (n=8). DPI significantly inhibited $O_2^-$ production in OM tissue segments of SS rats (n=8) compared with SS rats (n=8) as shown in the Table.

Effect of l-NAME on $O_2^-$ Production Determined by Chemiluminescence
To determine the relative contribution of NADPH oxidase and NOS to elevated $O_2^-$ production in SS rats, $O_2^-$ was measured using lucigenin (5 μmol/L) chemiluminescence in OM tissue segments incubated with DPI and l-NAME. $O_2^-$ production in OM tissue segments from hypertensive SS rats (n=6) was 36% higher than segments from SS-13BN rats (n=6) fed a 0.4% salt diet for 6 weeks, NOS activity was significantly higher in the SS-13BN (n=7) compared with SS rats (n=8) as shown in the Table.

Activities of NOS and ROS Scavenging Enzymes in the Renal OM

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>NOS Activity (pmol Citrulline/min/μg of Protein)</th>
<th>SOD Activity (mU/mg/μg of Protein)</th>
<th>Catalase Activity (nmol Formaldehyde/min/μg of Protein)</th>
<th>Glutathione Peroxidase Activity (nmol NADPH/mL/min/μg of Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl S</td>
<td>4.0 ± 0.6</td>
<td>56.5 ± 3.5</td>
<td>0.65 ± 0.11</td>
<td>4.16 ± 0.13</td>
</tr>
<tr>
<td>SS-13BN</td>
<td>6.6 ± 0.6*</td>
<td>78.5 ± 4.3*</td>
<td>1.18 ± 0.20*</td>
<td>3.54 ± 0.21*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs Dahl S rats.
significant changes occurred in SS rats, with BH$_4$ levels dropping 27% to 150±15 pg/mg of tissue, whereas BH$_2$ levels remained constant. BH$_4$ (239±29 pg/mg of tissue) and BH$_2$ (55±8 pg/mg of tissue) levels in SS-13BN rats remained relatively unchanged. The shift of BH$_4$ to BH$_2$ in the SS and the reduction of the ratio of BH$_4$/BH$_2$ are consistent with NOS uncoupling in this strain fed a 4% diet for 6 weeks.

**Total SOD, Catalase, and Glutathione Peroxidase Activity**

In addition to increased production of $O_2^-$ by NADPH oxidase and uncoupled NOS, reduced scavenging of $O_2^-$ by SOD or $H_2O_2$ by catalase also seem to contribute to elevated ROS levels in the OM of SS rats, as seen in Table 1. SOD and catalase activities were both significantly lower in SS rats (n=6), by 28% and 42%, respectively, compared with SS-13BN rats (n=6). Glutathione peroxidase activity, however, was 15% higher in SS rats (n=6).

**Discussion**

The present study examined whether NOS contributes to $O_2^-$ production in kidneys of hypertensive SS rats and tested the hypothesis that elevated BH$_2$ levels are importantly involved in this process. The results indicate that $O_2^-$ levels were elevated in the OM of SS rats and could be inhibited by L-NAME, whereas L-NAME increased $O_2^-$ production in consomic SS-13BN rats. SS rats also showed a reduced ratio of BH$_4$/BH$_2$ in the OM that was driven by increased concentrations of BH$_2$. Both the shift of BH$_4$ to BH$_2$ in the SS, together with inhibition of $O_2^-$ production, are consistent with NOS uncoupling.

This study provides the first in vivo evidence that it is the ratio of BH$_2$ to BH$_4$ that determines NOS uncoupling within the OM of the hypertensive kidney. Previous work demonstrating uncoupling was conducted in the aortic vasculature of rodents, and focused on the role of decreased BH$_4$. The highly sensitive and specific RP-HPLC method used in this study for the quantitation of tissue biopterins is an advance over those used previously. The commonly used method of Fukushima and Nixon calculates BH$_4$ indirectly by subtracting the levels of alkaline-stable biopterins from total biopterin. In contrast, BH$_4$ and BH$_2$ were individually measured in the present assay enabling the first direct measurements of these cofactors and enabled us to demonstrate that elevated BH$_2$ levels may drive uncoupling in vivo. S-BH$_4$ was validated for use as an internal standard in this assay because rodents do not contain S-BH$_4$. The highly sensitive and specific RP-HPLC method used in this study for the quantitation of tissue biopterins is an advance over those used previously. The commonly used method of Fukushima and Nixon calculates BH$_4$ indirectly by subtracting the levels of alkaline-stable biopterins from total biopterin. In contrast, BH$_4$ and BH$_2$ were individually measured in the present assay enabling the first direct measurements of these cofactors and enabled us to demonstrate that elevated BH$_2$ levels may drive uncoupling in vivo. S-BH$_4$ was validated for use as an internal standard in this assay because rodents do not contain S-BH$_4$. The highly sensitive and specific RP-HPLC method used in this study for the quantitation of tissue biopterins is an advance over those used previously. The commonly used method of Fukushima and Nixon calculates BH$_4$ indirectly by subtracting the levels of alkaline-stable biopterins from total biopterin. In contrast, BH$_4$ and BH$_2$ were individually measured in the present assay enabling the first direct measurements of these cofactors and enabled us to demonstrate that elevated BH$_2$ levels may drive uncoupling in vivo. S-BH$_4$ was validated for use as an internal standard in this assay because rodents do not contain S-BH$_4$. The highly sensitive and specific RP-HPLC method used in this study for the quantitation of tissue biopterins is an advance over those used previously. The commonly used method of Fukushima and Nixon calculates BH$_4$ indirectly by subtracting the levels of alkaline-stable biopterins from total biopterin. In contrast, BH$_4$ and BH$_2$ were individually measured in the present assay enabling the first direct measurements of these cofactors and enabled us to demonstrate that elevated BH$_2$ levels may drive uncoupling in vivo. S-BH$_4$ was validated for use as an internal standard in this assay because rodents do not contain S-BH$_4$. The highly sensitive and specific RP-HPLC method used in this study for the quantitation of tissue biopterins is an advance over those used previously. The commonly used method of Fukushima and Nixon calculates BH$_4$ indirectly by subtracting the levels of alkaline-stable biopterins from total biopterin. In contrast, BH$_4$ and BH$_2$ were individually measured in the present assay enabling the first direct measurements of these cofactors and enabled us to demonstrate that elevated BH$_2$ levels may drive uncoupling in vivo. S-BH$_4$ was validated for use as an internal standard in this assay because rodents do not contain S-BH$_4$. The highly sensitive and specific RP-HPLC method used in this study for the quantitation of tissue biopterins is an advance over those used previously. The commonly used method of Fukushima and Nixon calculates BH$_4$ indirectly by subtracting the levels of alkaline-stable biopterins from total biopterin. In contrast, BH$_4$ and BH$_2$ were individually measured in the present assay enabling the first direct measurements of these cofactors and enabled us to demonstrate that elevated BH$_2$ levels may drive uncoupling in vivo. S-BH$_4$ was validated for use as an internal standard in this assay because rodents do
not naturally produce S-BH₄, as confirmed by many studies in several tissues.²³ S-BH₄ would clearly not be a suitable standard in humans and other species where this analog naturally occurs.

It was also observed in this study that SS rats fed a 0.4% salt diet were on the verge of NOS uncoupling. Peroxynitrite and O₂⁻ have been reported to be powerful oxidants of BH₄ in vitro, so it would be anticipated that medullary O₂⁻ and BH₄ levels would be simultaneously elevated in the renal medulla of SS rats. However, this degree of oxidative stress was not sufficient to uncouple NOS in rats receiving the 0.4% diet, because l-NAME had no effect on O₂⁻ production in OM tissue segments of either SS or SS-13BN. When placed on a 4% salt diet, however, O₂⁻ levels increased in SS rats, which caused further oxidation of BH₄, such that the ratio of BH₄ to BH₂ approached 1. Studies using purified NOS by Vasquez-Vivar et al.²⁰ indicate that at this ratio of BH₂:BH₄, there is a critical shift away from the production of NO by NOS to the production of O₂⁻. This seems to be the case in vivo as well, because the present study showed that l-NAME inhibited O₂⁻ production in OM tissue segments, and the BH₂:BH₄ ratio nearly reached 1, indicating that NOS was uncoupled in hypertensive SS rats on a high-salt diet.

Unlike the SS rat, the SS-13BN control strain was able to compensate to the high-salt diet by increasing NOS activity, consistent with the response in other salt-insensitive strains.²⁶ The importance of this increased activity was shown when l-NAME significantly raised O₂⁻ production in OM tissue segments of SS-13BN rats fed a high-salt diet, indicating that NO was importantly involved in O₂⁻ scavenging. Although interstitial nitrate/nitrite levels in the SS-13BN were not measurably increased, the indirect nature of this measurement may not accurately reflect renal medullary NO levels.

The production of O₂⁻ by NOS seems to depend on the kindling radicals produced by NADPH oxidase, because, in the present study, DPI completely prevented the increase in OM O₂⁻ in SS rats fed a 0.4% or 4% salt diet while having no effect on SS-13BN rats, and apocynin did the same when infused chronically into the renal interstitium of these rat strains.¹⁴ If the salt-induced increase in NADPH oxidase radicals is not prevented, however, then NOS becomes uncoupled, and other enzymes become dysregulated, including SOD, catalase, and GPx. Similar results have also been seen in aortas of DOCA salt–induced hypertensive mice, suggesting that this may be a common mechanism in different forms of hypertension.⁸

The observed deficiency in catalase activity in hypertensive SS rats is novel and likely contributes to the elevated renal medullary H₂O₂ levels that we have reported previously.¹⁵ This is particularly relevant to the present study because H₂O₂ has been shown to participate in NOS uncoupling by downregulating dihydrofolate reductase, the enzyme that normally catalyzes the regeneration of BH₄ from its oxidized form, BH₂.²⁷ Elevated H₂O₂ levels may also initiate the epithelial-to-mesenchymal transition,¹⁵,²⁸ which leads to the tubulointerstitial injury that is so prominent in the kidneys of hypertensive SS rats.¹⁷

We conclude that there are several dysregulated pathways in hypertensive SS rats that could be contributing to elevated oxidative stress and salt-induced hypertension, including elevated NADPH oxidase activity, uncoupled NOS, diminished SOD, catalase and NOS activities, and that NOS uncoupling occurred because of a shift in the ratio of BH₄:BH₂ toward greater oxidation.

Perspectives
NADPH oxidase increasingly seems to be the initiating source of oxidative stress in various forms of hypertension. It is not yet clear, however, whether there is a common cause for the activation of this enzyme in these disparate models. Extensive work has been conducted elucidating the upstream and downstream signaling pathways linking angiotensin II and NADPH oxidase. Unfortunately, little is known about the links between renal Na⁺ handling and NADPH oxidase.

There are, however, some interesting clues. Inhibition of the Na'/H⁺ exchanger abolished the increase in O₂⁻ levels induced by elevation of metabolic activity in dissected renal medullary thick ascending limbs that were overloaded with NaCl, d-glucose, or triiodo-thyronine.²⁶ Studies in which the tubules were perfused rather than superfused showed similar results, in that increased Na⁺ delivery elevated O₂⁻ production, and this rise was inhibitable by ouabain.³⁰ Finally, the outflow of H⁺ mediated by Na'/H⁺ exchanger was shown to increase production of O₂⁻ via NADPH oxidase in medullary thick ascending limbs.³¹ It will be important in future work to follow up on these observations to elucidate the mechanisms by which NADPH oxidase is activated in salt-sensitive hypertension.

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Disclosures
None.

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NO Synthase Uncoupling in the Kidney of Dahl S Rats: Role of Dihydrobiopterin
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