Dahl Salt-Sensitive Rats Are Protected Against Vascular Defects Related to Diet-Induced Obesity

Andreas M. Beyer, Gabor Raffai, Brian Weinberg, Katherine Fredrich, Julian H. Lombard

Abstract—Obesity increases plasma renin activity and angiotensin II levels, leading to vascular damage, elevated blood pressure, diabetes mellitus, and renal damage. Because genetic deletion of crucial parts of the renin-angiotensin system protect against obesity-related cardiovascular defects, we hypothesized that Dahl salt-sensitive (SS) rats, a model of chronically low plasma renin activity and angiotensin II levels, would be protected against vascular defects during diet-induced obesity compared with SS.13BN consomic rats showing normal renin-angiotensin system regulation. We evaluated vascular function in middle cerebral arteries of SS or SS.13BN rats fed high-fat (45% kcal from fat) versus normal-fat diet for 15 to 20 weeks from weaning. Endothelium-dependent relaxation in response to acetylcholine (10^{-8} to 10^{-4} mol/L) was restored in middle cerebral arteries of high-fat SS rats versus normal-fat diet controls, whereas vasodilation to acetylcholine was dramatically reduced in high-fat SS 13BN rats versus normal-fat diet controls. These findings support the hypothesis that physiological levels of angiotensin II play an important role in maintaining normal vascular relaxation in cerebral arteries and suggest that the cerebral vasculature of the SS rat model is genetically protected against endothelial dysfunction in diet-induced obesity. (Hypertension. 2012;60:404-410.) • Online Data Supplement

Key Words: cardiovascular pathophysiology • renin • cardiovascular disease • endothelial dysfunction • microcirculation

The renin-angiotensin system (RAS) has long been associated with obesity and the metabolic syndrome. Multiple studies have shown that obesity is frequently accompanied by increased angiotensin II (Ang II) levels, elevated blood pressure, vascular dysfunction, renal damage, and diabetes mellitus. High-fat (HF) diet and obesity have been demonstrated to be associated with endothelial dysfunction, oxidant stress, and stroke in multiple experimental models and in human populations as well.5–11

By contrast, another risk factor for hypertension and vascular damage, increased dietary salt (NaCl) consumption, is characterized by reduced circulating Ang II and plasma renin activity (PRA).12–15 In a long-term follow-up study of salt sensitivity in humans, Weinberger et al16 found that individuals dying from cardiovascular causes had a significantly lower PRA than survivors or individuals dying from other causes. A commonly used experimental model of chronically lowered RAS activity is the Dahl salt-sensitive (SS) rat strain, which bears a striking similarity to SS hypertension in humans, particularly in the black population.

Endothelium-dependent vascular relaxation mechanisms that are absent even in normotensive SS rats fed a low-salt diet can be rescued either pharmacologically by chronic IV infusion of a subpressor dose of Ang II17 or genetically by introgressing chromosome 13 containing the renin gene from the Brown Norway (BN) rat into the SS genetic background.17–19 The resulting consomic strain (SS.13BN) exhibits normal regulation of the RAS and a significant reduction in the salt sensitivity of their blood pressure compared with the SS parental strain.19–21 SS.13BN consomic rats17–19 and congenic strains carrying the BN renin allele in the SS genetic background22 also exhibit reduced vascular oxidant stress17,22 and restored vascular relaxation in response to endothelium-dependent vasodilator stimuli.17–19,22

In human studies to investigate potential mechanisms of the paradoxical advantage of obese hypertensive patients for cardiovascular prognosis compared with lean hypertensives in some conditions (obesity paradox), Weber et al23 reported that obese hypertensive patients had an attenuated renin response to treadmill exercise compared with either lean hypertensive patients or lean control subjects. Because PRA and plasma Ang II levels are significantly lower in normotensive SS rats fed a low-salt diet compared with SS.13BN consomic rats and because SS rats have an impaired ability to regulate PRA normally,20,24,25 these animals may be protected against cardiovascular damage associated with obesity. We hypothesized that the reduced ability of SS rats to increase PRA and Ang II levels,20,24,25 as normally occurs...
with obesity, would protect these animals from the vascular defects and oxidant stress associated with diet-induced obesity (DIO) in animals showing normal regulation of the RAS.

In this study, we found that DIO in SS rats had the same effect as direct infusion of a low dose of Ang II in these animals, namely to restore endothelium-dependent relaxation of cerebral arteries in response to acetylcholine. The latter observation suggests that the SS rat may provide a novel experimental model that will help separate the systemic effects of altered Ang II levels from the direct effects of obesity on the vasculature.

Materials and Methods

Experimental Animal Groups
Male Dahl SS or SS BN/13 MCW consomic rats were placed on an HF diet (Research Diets D12451, 45% kcal from fat containing 10% Mineral Mix S10026 with final concentrations of 0.3% NaCl and 0.7% K) or normal-fat diet (NFD; 11.9% kcal from fat, 0.26% NaCl, 0.36% K) AIN 76 diet (Dyets, Inc, Bethlehem, PA) for 16 to 20 weeks after weaning (3-5 weeks of age). Body weight was monitored by biweekly weighing, and a subset of the animals was given losartan (20 mg/kg per day for 1 week) in the drinking water before and after treatment. As shown in Figure 1, SS rats fed an NFD diet were significantly lighter than SS-13BN animals fed NFD.

Cannulated Middle Cerebral Artery Preparation
On the day of the experiment, animals were anesthetized with a ketamine (78.0 mg/kg) and acepromazine (2.2 mg/kg) mixture. The brain was removed and immersed in physiological salt solution. The middle cerebral artery (MCA) was carefully excised, cannulated with glass microproppetes, pressurized to 80 mm Hg, and perfused and superfused with physiological salt solution (37°C) equilibrated with a 21% O2-5% CO2-74% N2 gas mixture, as described previously.18 Internal diameter was measured via television microscopy. Vessels that failed to develop intrinsic resting tone because of damage in isolation or mounting (<10% of all attempted experiments) were discarded. No outliers were excluded using the statistical 2 sigma test.

Response to Vasodilator Stimuli
Vascular diameter changes in response to the endothelium-dependent vasodilator agonist acetylcholine (ACh) and endothelium-independent NO donor sodium nitroprusside (MCA) were determined in each vessel. At the end of the experiment, resting tone and maximum diameter of the artery were assessed by superfusing the vessel with papaverine (100 μmol/L) or Ca2+-free physiological salt solution.

Arterial Blood Pressure Measurements
Chronic indwelling catheters were introduced via the femoral artery of anesthetized rats, as described previously.26,27 The animals were allowed a 3-day recovery period before beginning the experiment, and mean arterial pressure was measured in the conscious, freely moving rat.

Western Blots
The expression level of various proteins (Cu/Zn superoxide dismutase [SOD], MnSOD, endothelial NO synthase [eNOS], phosphorylated [p]-eNOS [ser 1177], Ang II type 1 receptor, and Ang II type 2) was evaluated by Western blotting in pooled samples of cerebral arteries including MCA and vessels of similar size from the ventral surface of the brain, as described in the online-only Data Supplement Methods section. Protein expression of Cu/Zn SOD, MnSOD, eNOS, and p-eNOS, was visualized by enhanced chemiluminescence SuperSignal West Pico (Thermo Scientific) and normalized to either β-actin (Cu/Zn SOD, MnSOD, and eNOS) or eNOS (p-eNOS).

Statistical Methods
Data are presented as mean±SEM. For all of the concentration-response curves, differences between groups at each concentration were determined using a 2-way, repeated-measures ANOVA. A post hoc Student-Newman-Keuls test was used to compare multiple means after ANOVA, and P<0.05 was considered to be statistically significant.

Results

Body Weight Progression and Food Intake
As shown in Figure 1, SS rats fed an NFD diet were significantly lighter than SS-13BN rats fed an NFD diet. SS HF rats gained weight faster and were significantly heavier than SS rats fed an HF diet. The weight of SS rats fed an HF diet was comparable to that of SS-13BN animals fed NFD. Conscious blood pressure was significantly elevated in the SS HF group (Figure 2), whereas blood pressures in the other groups were not significantly different.

Cerebral Vascular Function
Endothelium-dependent dilation to ACh that was absent in MCA of SS rats fed an NFD diet was restored in SS rats ingesting an HF diet for 15 to 20 weeks (Figure 3), but HF diet impaired ACh-induced dilation of MCA in SS-13BN controls. Figure 4 summarizes the effect of Ang II type 1 receptor blockade with losartan and acute free radical scavenging with Tempol in the physiological salt solution on the responses to ACh in MCA from the various groups. Losartan eliminated the restored dilation to ACh in MCA from SS rats fed an HF.

Figure 1. Body weight progression and food intake of salt-sensitive (SS)-13 Brown Norway (BN) vs SS rats fed high-fat (HF) or normal-fat diet (NFD) diet. Comparison of increase in body weights over the course of treatment with either HF or NFD (A) and food intake in grams per day (B). *P<0.05 SS HF vs NFD control. #P<0.05 vs SS-13BN on same diet. Data are expressed as mean±SEM for n=8. A, ●, SS-13BN HF; ○, SS HF; ▼, SS-13BN NFD; △, SS NFD. B, ■, NFD; □, HF.
diet and ameliorated endothelial dysfunction in MCA from SS.13BN rats fed an HF diet, with no effect on vessel responses to ACh in MCA of SS or SS.13BN rats fed NFD. Tempol restored ACh-induced dilation of MCA from SS rats fed an NFD and SS.13BN rats fed an HF diet, with no effect on responses to ACh in MCA of SS rats fed an HF diet or SS.13BN rats fed an NFD.

The protective effect of HF diet to restore endothelium-dependent dilation in SS rats was likely mediated by an increase in NO levels, because preincubation with N\textsuperscript{\textdagger} nitro-l-arginine methyl ester (100 \textmu mol/L) abolished vasodilation to ACh in SS rats fed an HF diet (Figure S1, available in the online-only Data Supplement). Endothelium-independent relaxation to the NO donor DETA NONOate was similar in all of the experimental groups (Figure 3).

**Evaluation of Renal Damage in Dahl SS and SS.13BN Rats**

Because the Dahl SS rat is a commonly used model for renal hypertension, we evaluated renal function by measuring microalbumin in the urine and more protein casts in the renal tubes histologically. HF diet led to a significant increase in urinary protein and microalbuminuria in both groups. Proteinuria and microalbuminuria were significantly greater in SS rats versus SS.13BN rats fed an HF diet. Protein casts (Figures 5C and S2) were significantly higher in renal tubules of SS rats fed an NFD and HF diet compared with SS.13BN rats fed the same diet. The latter findings are all consistent with the previously documented renal protective effect of substituting BN chromosome 13 into the SS genetic background, as is the lack of a blood pressure increase in SS.13BN rats fed an HF diet versus SS, where arterial pressure was significantly elevated with an HF diet concomitant with elevated protein and microalbumin in the urine and more protein casts in the renal tubules.

**Protein Expression Using Western Blot**

CuZn SOD expression in cerebral arteries of SS rats fed an NFD was significantly lower than that of SS.13BN controls (Figure 6A). HF diet led to a significant increase in CuZn SOD expression in arteries of SS rats but not SS.13BN controls. HF diet also tended to increase MnSOD expression in arteries of SS rats (Figure 6B). In contrast to the vascular phenotype, total eNOS expression was significantly reduced in the SS HF group (Figure 6C), although phosphorylation of eNOS at Ser 1177, which activates eNOS-dependent NO production, appeared to be higher in SS rats fed an HF diet compared with SS control rats fed an NFD (Figure 6D).

Ang II type 1 receptor expression was significantly reduced by HF diet in SS rats (Figure 6E), whereas expression of the Ang II type 2 in the cerebral vasculature of SS HF rats tended to be higher than that of control animals fed an NFD (Figure 6F). The restored dilation to ACh in conjunction with a possible increase in the expression of the Ang II type 2 receptor in SS rats fed an HF diet is consistent with the results of a recent study\textsuperscript{28} showing that chronic activation of the Ang II type 2 receptor restores endothelium-dependent vascular relaxation in mesenteric arteries of Sprague-Dawley rats fed a high-salt diet (another model of endothelial dysfunction and increased oxidant stress).

**Discussion**

A number of studies\textsuperscript{2,29} have shown that elevated levels of circulating Ang II are associated with increased body weight, body fat, and plasma insulin levels. Relevant to its cardiovascular effects, obesity is closely associated with elevated blood pressure, decreased endothelial function, heart failure, and other cardiovascular risks, which may be caused, at least in part, by an excessive activation of the RAS.\textsuperscript{30-32}

In mice, genetic deletions of crucial parts of the RAS have a protective effect against DIO.\textsuperscript{33} Mice lacking the renin substrate angiotensinogen (Agt) are lean and resistant to DIO.\textsuperscript{34} Ren\textsuperscript{\textdagger} knockout mice show increased energy expenditure, dietary fat wasting,\textsuperscript{32} and similar plasma levels of adiponectin (a commonly used marker for adiposity) as wild-type mice, with no effects on blood pressure, food intake, blood glucose, and plasma creatinine.\textsuperscript{33} Ang II type 1A receptor knockout mice (Agtr1a\textsuperscript{\textdagger}) exhibit increased Ang II levels as part of the feedback loop but are also lean, resistant to DIO, and show high metabolic rate (in line with Ang II levels increasing metabolism).\textsuperscript{35}
We reported previously that Ang II suppression with high-salt diet in Sprague-Dawley rats increases vascular oxidant stress, and impairs endothelial-dependent vascular relaxation. Dahl salt-sensitive rats are a commonly used model to study the effects of high-salt diet and low renin activity on the cardiovascular and renal system. Dahl SS rats fed a normal-salt diet are exposed to chronically low levels of circulating Ang II as a result of their inability to regulate PRA normally. Decreased activity of the RAS is associated with a protective effect against the effects of obesity, and renin inhibitors (which lower PRA and plasma Ang II levels) have been shown to increase the bioavailability of NO, decrease vascular oxidative stress, and protect against atherosclerosis with obesity in Watanabe heritable hyperlipidemic rabbits.

SS rats show increased oxidant stress, reduced antioxidant defense mechanisms, and impaired vascular relaxation to multiple vasodilator stimuli, even when they are normotensive and maintained on a normal-salt diet. As noted above, impaired vascular relaxation and endothelial dysfunction in SS rats can be rescued not only pharmacologically by chronic IV infusion of a subpressor dose of Ang II but also genetically by introgression of BN chromosome 13 containing a normally functioning renin allele into the SS genetic background (SS.13BN consomic rat) to restore normal circulating levels of Ang II.

Because of the chronically suppressed RAS in SS rats, we hypothesized that the effects of obesity-related increases in RAS activity on vascular function would be ameliorated in SS rats but would cause a significant decrease in vascular function in SS.13BN rats, which are protected against salt-induced increases in arterial blood pressure but have a higher basal RAS activity. Consistent with this hypothesis, SS rats fed an HF diet for 15 to 20 weeks after weaning showed an improved endothelium-dependent dilation to ACh compared with SS rats fed an NFD diet and HF-fed SS-13BN consomic control rats (~98% genetically identical to SS but exhibiting normal activity of the RAS). By contrast, HF diet caused an impaired endothelium-dependent dilation to ACh in SS.13BN consomic rats.

The hypothesis that Ang II plays a crucial role in restoring cerebral vascular relaxation in SS rats fed an HF diet is supported by the observation that treatment of the animals with the Ang II type 1 receptor antagonist losartan blocked the protective effect of the HF diet to restore vascular relaxation. By contrast, in the SS.13BN rats, losartan tended to restore vascular relaxation to ACh in HF-fed animals and had no effect on endothelium-dependent dilation to ACh in SS.13BN rats fed an NFD.

Interestingly, other effects of DIO (e.g., increased BW and a further increase in renal defects) are still observed in both

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**Figure 4.** Effect of Tempol (A and B) or losartan (C and D) on response of cannulated middle cerebral artery (MCA) to acetylcholine (Ach) in salt-sensitive (SS) and SS.13 Brown Norway (BN) rats fed a normal-fat diet (NFD; A and C) or high-fat (HF) diet (B and D). Untreated control responses were compared in Figure 3. Data are expressed as mean±SEM for n=6, except for SS.13BN+Tempol (n=2) on NFD. *P<0.05 SS control vs SS treated (Tempol or losartan); #P<0.05 SS.13BN control vs SS.13BN treated (HF Tempol, losartan NFD and HF diet); ##P<0.05. SS treated vs SS.13BN treated. Because of the small N, no statistical comparisons were made with SS.13BN+Tempol. Previous studies (Reference 19) have shown that ACh-induced dilation in MCA of SS.13BN on NFD is unaffected by Tempol. SS; SS.13BN; Tempol; SS.13BN Tempol.

**Figure 5.** Effect of high-fat (HF) diet vs normal-fat diet (NFD) on microalbumin (A), urinary protein (B), and protein casts in renal tubules (C) of salt-sensitive (SS) vs SS.13 Brown Norway (BN) rats. Protein casts were quantified from Figure S2. *P<0.05 vs NFD control and #P<0.05 vs SS.13BN on same diet (n=8 per group). NFD, HF.
rat strains, in spite of the improved vascular function in SS rats fed an HF diet. Glucose and insulin tolerance tests and measurements of fasting blood glucose levels revealed no significant difference between any of the groups (data not shown), and HF diet did not have a protective effect on renal function in either strain.

In summary, we found that SS rats, a model of low RAS activity, are protected against the deleterious effects of obesity on the cerebral vasculature that are ordinarily mediated via Ang II and that DIO actually restores endothelium-dependent dilation to Ch that is normally absent in MCA of SS rats. The findings of the present study support the concept that physiological levels of Ang II play an important role in maintaining normal vascular relaxation mechanisms by showing that a genetic rodent model of low renin, salt-sensitive hypertension exhibits enhanced endothelial function with a prolonged HF diet. This mechanism is likely mediated, at least in part, by increased expression of superoxide dismutase; and changes in the expression of the Ang II type 1 and possibly Ang II type 2 receptors for Ang II occur with ingestion of an HF diet. The effects of Ang II type 1 receptor blockade with losartan are also consistent with a role for Ang II both in leading to endothelial dysfunction in HF-fed SS.13BN rats and in contributing to the protective effect of lower Ang II levels to restore vascular relaxation in HF-fed SS rats. Taken together, the findings of the present study provide further support for the hypothesis that proper levels of circulating Ang II are critically important in maintaining vascular function, as both lowered Ang II levels (high-salt diet, SS rats) and increased levels of Ang II (severe Na+ restriction, high-dose Ang II infusion, and obesity) lead to vascular defects such as endothelial dysfunction. Overall, the results of this study underscore the need for further study of the role of the RAS in obesity-mediated vascular changes and suggest that the Dahl SS rat may be an excellent model system for this undertaking.

**Perspectives**

The findings of the present study suggest that the SS rat is genetically protected against endothelial dysfunction in DIO despite increased body weight, elevated blood pressure, and reduced renal function. The observation that HF diet has a protective effect against endothelial dysfunction is, at first glance, surprising. However, the concept that mild obesity has a protective effect on cardiovascular phenotypes is not without precedent. For example, leptin, a marker for adiposity, is an NO-independent coronary artery vasodilator in humans; and Okere et al reported that HF diet prevents the hypertrophic response to hypertension and improves the contractile performance of the heart in Dahl SS rats.

The current findings may be especially relevant to findings in human patient populations with established cardiovascular disease. Although obesity is a known risk factor in the pathogenesis and progression of cardiovascular disease, there are numerous reports of an “obesity paradox,” where obesity and higher body mass index are associated with lower mortality in patients with established cardiovascular disease. Although the mechanism of this phenomenon remains elusive, attenuated neurohumoral responses, including reduced activation of the RAS, have been suggested as a possible factor contributing to the obesity paradox. In this regard, it would be interesting to determine whether obesity or higher body mass index has any protective effect in individuals with low PRA, who exhibit a significantly higher mortality from cardiovascular causes than survivors in long-term follow-up studies.

**Acknowledgments**

We thank the Department of Physiology assay and microscopy core facilities for measurement of several parameters during the develop-
Sources of Funding

National Institutes of Health grants HL65289 (to J.H.L.), HL-72920 (to J.H.L.), and HL-92026 (to J.H.L.).

Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- This study shows that HF diet to produce DIO results in a paradoxical restoration of endothelial function in Dahl SS rats that can be prevented by Ang II type 1 receptor blockade with losartan.

**What Is Relevant?**

- The present study supports the hypothesis that normal physiological levels of Ang II play a crucial role in maintaining normal vascular relaxation, whereas elevated Ang II levels contribute to endothelial dysfunction.

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**Summary**

Ingestion of an HF diet to produce DIO ameliorated endothelial dysfunction in Dahl SS rats and abrogated endothelium-dependent dilation to ACh in SS.13BN consomic rats showing normal regulation of PRA. The protective effect of HF diet to restore vascular relaxation in the SS rats was prevented by Ang II type 1 receptor blockade with losartan.
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Hypertension. 2012;60:404-410; originally published online June 18, 2012;
doi: 10.1161/HYPERTENSIONAHA.112.191551
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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Dahl Salt Sensitive Rats are Protected Against Vascular Defects Related to Diet-Induced Obesity

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Active Tone and Maximum Diameter: MCA from SS.13^{BN} rats fed HF diet developed significantly less myogenic tone (33±4.1%, n=10) than SS.13^{BN} rats fed NFD (46±2.1%, n=6). Myogenic tone in MCA of SS.13^{BN} rats fed HF diet was comparable to that in SS animals fed either diet (32±4.8%, n=10 NFD and 32±3.2%, n=12 HF diet). There was no significant difference in maximum diameter of MCA from SS or SS.13^{BN} rats fed either diet (not shown), indicating that any changes in vessel responses to vasodilator stimuli were not due to structural remodeling of the vessel.
SUPPLEMENTAL MATERIALS AND METHODS

Cannulated Middle Cerebral Artery (MCA) Preparation: On the day of the experiment, animals were anesthetized with a ketamine (78.0 mg/kg) and acepromazine (2.2 mg/kg) cocktail and the brain was removed and immersed in physiological salt solution (PSS) having the following ionic composition (mM): NaCl (119.0), KCl (4.7), CaCl₂ (1.6), NaH₂PO₄ (1.18), MgSO₄ (1.17), NaHCO₃ (24.0), D-glucose (5.5), and ethylenediaminetetraacetic acid (EDTA) (0.03). The MCA was carefully excised under a dissecting microscope (Leica; Buffalo, NY), cannulated with glass micropipettes (80-120 µm; FHC, Brunswick, ME) at the proximal and distal ends and extended to its approximate in situ length. Side branches were ligated to prevent leaks and to allow the vessel to be pressurized. The vessel was continuously perfused and superfused with PSS (37°C) that was equilibrated with a 21% O₂-5% CO₂-74% N₂ gas mixture, and the intraluminal pressure was maintained at 80 mmHg to approximate in vivo conditions. Internal diameter of the artery was measured using television microscopy and a video micrometer (model IV-550, FOR-A Company; Tokyo, Japan). Vessels lacking intrinsic resting tone were excluded from analysis.

Response to Vascular Stimuli: Vascular diameter changes in response to the endothelium-dependent vasodilator agonist ACh (10⁻⁸ to 10⁻⁴ M) and endothelium-independent NO donor deta-NONOate (10⁻⁹ to 10⁻⁵ M) was determined in each vessel. At the end of the experiment, resting tone and maximum diameter of the artery were assessed by superfusing the vessel with papaverine (100 µM) or Ca²⁺-free PSS having the following composition (mM): NaCl (119.0), MgCl₂ (20.0), KCl (4.7), NaH₂PO₄ (1.18), MgSO₄ (1.17), NaHCO₃ (24.0), D-glucose (5.5), and ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (2.0). Pharmacological agents to inhibit NOS enzymes (L-NAME 10⁻⁴ M) or scavenger of free radicals (Tempol, 10⁻⁶ mol liter⁻¹) used directly in the organ bath. Losartan (1 mg/mL) was administered to the whole animal via drinking water for 1 week prior to vascular studies.

Resting tone (T) in % was calculated as [(Dₘₐₓ-Dₗₑ₉)/Dₘₐₓ] x100, where Dₘₐₓ is the maximum diameter in Ca²⁺ free solution and Dₗₑ₉ is the resting control diameter.

Arterial Blood Pressure Measurements: For measurement of arterial blood pressures, rats were anesthetized with an intramuscular injection containing ketamine (78.0 mg/kg) and acepromazine (2.2 mg/kg). Chronic indwelling catheters were introduced via the femoral artery using previously described procedures.¹,² The animals were allowed a 3-day recovery period before beginning the experiment, and mean arterial pressure was measured in the conscious, freely moving rat.

Evaluation of Renal Function and Structure: To evaluate renal function, urine was collected for two consecutive days and nights using metabolic cages for rats in each group. Urine was frozen for later evaluation of microalbumin and protein levels by the department’s core assay facility.
Renal structure was evaluated using sections from formaldehyde fixed kidneys isolated the day of the vascular experiment. The kidneys were placed in 4% formaldehyde for 48 hours and then transferred for 48+ hours to 10% glucose. Sections were trichrome stained by the physiology microscopy core faculty.

**Western Blots:** The expression level of various proteins [Cu/Zn SOD, MnSOD, eNOS, p-eNOS (ser 1177), AT₁R, and AT₂R] was evaluated by Western blotting in pooled samples of cerebral arteries including MCA and vessels of similar size from the ventral surface of the brain. Briefly, samples containing 2.5 μg of protein were separated by 4-20% SDS-PAGE, transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibodies in 2% nonfat dry milk in TBST buffer [Cu/Zn SOD (Stressgen) at 1:6000; Mn SOD (Assay Design, Ann Arbor, MI, SOD-111) at 1:8000; eNOS (BD Biosciences) at 1:3000, p-eNOS (BD Biosciences) at 1:1000; AT₁R (Sigma) at 1:800; AT₂R (Sigma) at 1:1000, β-Actin (Sigma) at 1:35,000] at 4°C overnight. The membrane was then incubated with secondary HRP-conjugated antibody in 2% nonfat dry milk in TBST buffer at room temperature for 2 hours (goat anti-rabbit 1:6000 for Cu/Zn SOD and MnSOD; goat anti-mouse 1:35,000 for β-actin; goat anti-mouse 1:2000 for p-eNOS), and protein expression of Cu/Zn SOD, MnSOD, eNOS, and p-eNOS, was visualized by enhanced chemiluminescence SuperSignal West Pico (Thermo Scientific).
SUPPLEMENTAL REFERENCES


Supplemental Figure S1. Effect of NOS inhibition via L-NAME (100 µmol/L) on ACh-mediated endothelial dilation in SS NFD and SS HF diet rats. Data summarized as mean change in resting diameter ± SEM for “5” vessels, paired. * P<0.05 L-NAME treated vs. nontreated control.
Supplemental Figure S2. Increased protein casts in SS Rats. Sections are stained with trichrome staining, where blue staining shows protein casts in the renal tubules.