Changing standard chow diet promotes vascular NOS dysfunction in Dahl S rats

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Abstract

We hypothesized that vascular nitric oxide synthase (NOS) function and expression is differentially regulated in adult Dahl S rats maintained on Teklad or AIN-76A standard chow diets from 3 to 16 weeks old. At 16 weeks old, acetylcholine (ACh)-mediated vasorelaxation and phenylephrine (PE)-mediated vasoconstriction in the presence and absence of nitric oxide synthase (NOS) inhibitor, L-NAME, was assessed in small resistance mesenteric arteries and aortae. Rats maintained on either diet throughout the study had similar responses to ACh and PE in the presence or absence of L-NAME in both vascular preparations. We reasoned that changing from one diet to another as adults may induce vascular NOS dysfunction. In the absence of L-NAME, small arteries from Teklad-fed rats switched to AIN-76 diet and vice versa had similar responses to ACh and PE. Small arterial NOS function was maintained in rats switched to AIN-76A from Teklad diet. Whereas, NOS function in response to ACh and PE was lost in the small arteries from rats changed to Teklad from AIN-76A diet. This loss of NOS function was echoed by reduced expression of NOS3 as well as phosphorylated NOS3. The change in NOS phenotype in the small arteries was observed without changes in blood pressure. Aortic responses to ACh or PE in the presence or absence of L-NAME were similar in all diet groups. These data indicate that changing standard chow diets leads to small arterial NOS dysfunction and reduced NOS signaling predisposing Dahl S rats to vascular disease.

Keywords: standard diet, Dahl rat, L-NAME, nitric oxide synthase, vascular reactivity
**Introduction**

The nitric oxide synthase (NOS) pathway plays an obligatory role in vascular tone homeostasis (1). Loss of NOS signaling results in a loss of tonic vasorelaxation favoring greater vasoconstriction (11, 26, 42) and is linked to increased risk of vascular disease (3, 20, 43). Humans that present increased blood pressure to salt have a greater disposition to vascular disease risk even under normotensive conditions (44, 45). A prototypical model to study vascular dysfunction is the Dahl salt-sensitive (Dahl S) rat. In this rat strain, a high-salt diet induces robust hypertension and vascular dysfunction (5, 6, 14, 25, 28, 32, 37). Intriguingly under normotensive conditions without high-salt diet, Dahl S rats have lower vascular NOS expression (30) and greater blood pressure responsiveness to bolus doses of phenylephrine (PE; α₁-specific agonist) compared to the genetic salt-resistant control strain (10). These data suggest that a genetic predisposition to salt-sensitivity *per se*, not frank hypertension, enhances vascular disease risk.

Our laboratory is interested in understanding mechanisms that predispose salt-sensitive individuals to vascular disease using the Dahl S model. Previous investigations showed that promotion of salt-sensitive hypertension in adult Dahl S rats is dependent on the type of weaning diet (27). This led us to investigate whether vascular NOS function and expression are sensitive to the choice of standard chow diets. The two popular commercial suppliers of Dahl S rats, Harlan Laboratories (www.harlan.com) and Charles River Laboratories (www.criver.com), each maintains their Dahl S colonies on different commercially-available standard chows. Harlan uses Teklad diet and Charles River utilizes American Institutes of Nutrition (AIN) purified diet. The standard chow at many institutions is Teklad diet; therefore, some experimental designs would incorporate changing the standard chow diet during adulthood once the rats are housed at the investigator’s institution. We reasoned that changing between standard chow diets may differentially regulate vascular NOS function. Thus, we
designed experiments to test the hypothesis that vascular NOS function and expression are
differentially regulated in adult Dahl S rats on Teklad 8604 or AIN-76A standard chow diets.
For this purpose, a colony of Dahl S rats was generated at Georgia Health Sciences University
to maintain a comparable stable environment. Male offspring were weaned on Teklad 8604 or
AIN-76A standard chow diet and were either maintained on the same weaning diet or switched
to the other standard chow diet at 12 weeks old. Mesenteric arterial and aortic reactivity as well
as NOS function and expression were assessed at 16 weeks old.
Materials and Methods

Animal Model

Dahl S rat breeders were purchased from Charles River Laboratories (Wilmington, MA) and placed on Teklad 8604 diet upon arrival at Georgia Health Sciences University (GHSU). First-generation Dahl S rats from 6 breeding pairs were used in this study. A genome-wide scan using microsatellite primers, as detailed by Moreno et al. (29) that were specific to Dahl DNA confirmed the genetic background of the breeders. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal use protocols were approved by the Institutional Animal Care and Use Committee at GHSU.

At weaning (3 weeks old), a subset of male pups from each breeding pair, were fed Teklad 8604 rodent diet (Teklad; Madison, WI) or AIN-76A purified diet (TestDiet; Richmond, IN) ad libitum. The Teklad diet consisted of calories from: 33% protein, 53% carbohydrates, and 14% fat and 3.93 kcal/g gross energy. The AIN-76A diet consisted of calories from: 19% protein, 69% carbohydrates, and 12% fat and 3.84 kcal/g gross energy. Both the Teklad and AIN-76A diets contained 0.4% NaCl with similar vitamin compositions. All rats were given tap water ad libitum.

At 12 weeks old, Teklad-weaned or AIN-weaned rats underwent a diet-switch protocol where a subset of Teklad rats was switched to AIN and vice versa. Importantly, rats generated from each breeding pair were included in each of the 4 diet groups (Figure 1A). Weekly body weights, food, and water intakes were assessed on all rats. At 16 weeks old, rats were euthanized (Nembutal; Abbott Laboratories, Abbott Park, IL; 0.5 mg/kg). Kidney, heart, epididymal adipose tissue weights and tibia lengths were assessed. Blood was collected in EDTA (Sigma, St. Louis, MO)-primed syringes, spun at 3000xg for 10 minutes, and snap-
frozen in liquid N₂. Mesenteric arteries and aortae were isolated, cleaned for ex vivo vascular reactivity analysis or snap-frozen in liquid N₂ for Western blotting as described below.

Telemetry Hemodynamic and Activity Measurements

Rats were implanted with telemetry transmitters (Data Sciences International; St. Paul, MN) at 11 weeks old as described previously (16). Rats recovered from surgery ~1 week while having free access to tap water and their respective diet. From 12-16 weeks old, the diet-switch protocol was performed (Figure 1A) whilst mean arterial blood pressure measurements were collected every tenth minute. Blood pressure is reported as a 24 hour average or 12 hour average.

Urine Collection

At 16 weeks old, rats were placed in metabolic cages to collect 24 hour urine volumes. Urines were snap frozen in liquid N₂ and stored at -80°C until analyzed. Urinary Na excretion was determined (EasyLite; Medica Corporation; Bedford, MA) with data expressed as mEq/24h. Urinary total protein excretion was quantified using standard protein assay (BCA assay, Bio-Rad; Hercules, CA).

Vascular Reactivity

Thoracic aortae and third-order mesenteric arteries were cleaned of adherent fat and cut into concentric rings and mounted on pins and chucks, respectively, for wire myography (Danish Myo Technology A/S; Aarhus, Denmark) as previously described (24). Aortic and small mesenteric artery segments were constricted with 1 μM and 2 μM phenylephrine (PE), respectively, followed by evaluation of vasorelaxation with cumulative-concentration response curves to acetylcholine (ACh; 1 x 10⁻⁹ M to 3 x 10⁻⁶.⁵ M for mesenteric arteries and 1 x 10⁻⁹ M to 3 x 10⁻⁴.⁵ M for aortae) then to sodium nitroprusside (SNP; 1 x 10⁻¹⁰ M to 3 x 10⁻⁶.⁵ M for mesenteric arteries and 1 x 10⁻¹⁰ M to 3 x 10⁻⁵.⁵ M for aortae) in the same artery segment. Vasorelaxation data are presented as relaxation (% PE constriction) as analyzed by the
equation \(((\text{maximum PE response} - \text{ACh response}) / (\text{maximum PE response} - \text{baseline prior to PE constriction})) \times 100\). Vasoconstriction was assessed with PE (1 x 10^{-9} M to 3 x 10^{-5} M) followed by KCl concentration-response curve (8 x 10^{-3} M to 100 x 10^{-3} M). Constriction responses are presented as % increase in force as analyzed by the equation \(((\text{response to vasoconstrictor} - \text{baseline prior to constriction}) / \text{baseline prior to constriction}) \times 100\). Rings were incubated in the presence or absence of the non-specific NOS inhibitor \(N_\omega\text{-Nitro-L-arginine methyl ester (L-NAME; 100µM; Sigma)}\) for 15 minutes prior to construction of response curves. Maximum response and sensitivity to the vasoactive agonists are expressed as \(E_{\text{max}}\) and \(\log EC_{50}\), respectively. \(\log EC_{50}\) was determined with GraphPad Prism software (La Jolla, CA).

**Western Blotting**

In a subset of rats from each diet group, whole mesenteric arterial beds were homogenized in 400µl ice-cold lysis buffer (50 mM Tris, 0.1 mM EDTA disodium salt, 0.1 mM EGTA, 0.1 mM sucrose, 0.1% BME, 10% glycerol, 2 µM leupeptin, 2 µM pepstatin A, 1mM phenylmethylsulfonyl fluoride, 0.1% aprotinin; 20 mM NaVO₃; pH 7.4); PhosSTOP tablets were used according to manufacturer’s instructions (Roche Diagnostics; Indianapolis, IN). Homogenates were spun at 10,000 rpm at 4°C for 5 minutes and supernatants isolated. Protein concentrations of supernatants were determined (BCA assay, Bio-Rad). 30 µg of protein was separated via 8% SDS-PAGE, transferred to PVDF membranes, and probed using anti-NOS1, anti-NOS3, anti-NOS3-phosphoserine1177 (all NOS antibodies at 1:500; BD Biosciences; San Jose, CA), and β-actin (1:10,000; Sigma). NOS antibodies were visualized with goat anti-mouse (1:1000; Invitrogen; Carlsbad, CA) and β-actin detected with goat anti-rabbit (1:10,000; Invitrogen) secondary antibodies using the Odyssey Infrared Imaging System (LI-COR Biosciences; Lincoln, NE). Analysis of NOS expression was normalized to β-actin. Further analysis of NOS3-phosphoserine1177 was normalized to NOS3 expression.
Plasma nitrite/nitrate measurement

Plasma was extracted using 1:1 (v:v) HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ) followed by centrifugation at 10,000 x g for 5 minutes at 4°C to evaluate nitrite and nitrate levels by HPLC (ENO-20; EiCom Corporation, Kyoto, Japan) as previously described (17).

Statistical Analyses

All data are expressed as mean ± standard error. Statistical significance was defined as $P<0.05$ as determined by Student's t-test or two-way ANOVA where indicated (GraphPad Prism).
Results

Metabolic Parameters

Figure 1A shows the experimental design and nomenclature utilized for the study. Dahl S rats fed either Teklad or AIN standard chow diets from 3 weeks until 16 weeks old gained weight similarly and had comparable tibia lengths demonstrating that neither standard diet differentially affected rat growth (Figure 1B; Table 1). Food and water intakes were also similar at 16 weeks old (Table 1). Heart weight and epididymal fat mass were similar in all diet groups (Table 1); however, kidney weights were significantly smaller in the groups raised on the AIN diet versus the Teklad with no difference between the other groups (Table 1).

A subset of each weaning-diet group underwent a diet switch protocol from 12-16 weeks old (i.e., [Teklad diet-fed rodents switched to AIN diet at 12 weeks old, referred to as Teklad→AIN, and vice versa]). Body and organ weights were similar to respective weaning diet counterparts at 16 weeks old (Table 1). No statistically significant difference in food or water intake was observed at 16 weeks old between the 4 diet groups (Table 1).

Hemodynamic and activity measurements

At 16 weeks old, 24h MAP and heart rate were similar in the non-switched weaning diet groups (Teklad or AIN); the trend for increased 24h MAP in the diet-switch groups (Teklad→AIN or AIN→Teklad) was not significant (Figure 2A). Additionally, the tendency for 24h MAP to increase from 12 weeks old to 16 weeks old was not statistically different between the 4 diet groups (Figure 2A). At 16 weeks old, 24h heart rate was similar in all diet groups (Figure 2B). Circadian rhythms of 12h MAP (Figure 2C) and heart rate (Figure 2D) were similar during the last 3 days of the diet-switch protocol (at 15 weeks, 5 and 6 days old and at 16 weeks old).
**Vasorelaxation**

Cumulative-concentration response curves to acetylcholine (ACh) were generated to assess endothelial function in third-order small resistance mesenteric arteries and thoracic aortae. No difference in maximum relaxation ($E_{\text{max}}$, Table 2) or sensitivity ($\log EC_{50}$, Table 2) was detected between weaning diet groups or diet switch groups in small mesenteric arteries as well as the response to the exogenous NO donor, SNP, between all 4 groups of Dahl S rats (Table 2).

To assess NOS function, ACh-mediated relaxation curves were generated in the presence of the non-specific NOS inhibitor, L-NAME. L-NAME significantly reduced sensitivity to ACh in Teklad (Figure 3A; Table 2), AIN (Figure 3B; Table 2), and Teklad→AIN (Figure 3C; Table 2) diet groups; however, the trend for L-NAME pretreatment to reduce ACh sensitivity in mesenteric artery segments from AIN→Teklad Dahl S rats was not statistically significant (Figure 3D; Table 2).

Aortic vasorelaxation to ACh and SNP was similar in all 4 diet groups and L-NAME totally blocked the ACh response (Table 3).

**Vasoconstriction**

Cumulative-concentration response curves to PE were used to assess vasoconstriction in third-order mesenteric arteries. PE-induced vasoconstriction was similar in all 4 diet groups (Table 4). In addition, KCl-induced vasoconstriction was similar in mesenteric arteries isolated from Teklad-fed, AIN-fed, and Teklad→AIN Dahl S rat diet groups (Table 4). However, the KCl response was significantly less in AIN versus all other diet groups (Table 4).

L-NAME pretreatment significantly increased sensitivity to PE-induced constriction in mesenteric arteries isolated from Teklad-fed (Figure 4A; Table 4), AIN-fed (Figure 4B; Table 4), and Teklad→AIN (Figure 4C; Table 4) Dahl S rats. Whereas, L-NAME had no significant
effect on the PE-induced vasoconstriction of mesenteric arteries from AIN→Teklad Dahl S rats (Figure 4D; Table 4).

Aortic response to PE and KCl was similar in all 4 diet groups (Table 5). L-NAME had no significant effect on aortic response to PE in any of the 4 diet groups (Table 5).

**NOS protein expression**

NOS3 protein expression was significantly reduced in small mesenteric arteries from AIN and Teklad→AIN compared to Teklad (Figure 5A and 5B). Moreover, NOS3 protein expression was further reduced in small mesenteric arteries from AIN→Teklad compared to AIN (Figure 5A and 5B). The NOS3-p1177 expression (Figure 5C and 5D) was similar in small mesenteric arteries from Teklad and AIN. NOS3-p1177 expression in arteries from Teklad→AIN was significantly higher compared to arteries from Teklad (Figure 5C and 5D), while the arteries from AIN→Teklad demonstrated a significantly reduced NOS3-p1177 expression compared to AIN (Figure 5C and 5D). NOS1 expression was not significantly different between the 4 diet groups (data not shown).

**Plasma Nitrite/Nitrate**

Nitrite levels were similar in all 4 diet groups (Figure 5E). Moreover, nitrate levels were similar in non-switched weaning diet groups (Figure 5F). Interestingly, nitrate levels were increased following the Teklad→AIN diet switch whereas no change was detected in the AIN→Teklad diet-switch group (Figure 5F).
Discussion

The principal finding of this study is that changing standard chow diets in adult Dahl S rats can lead to alterations in the NOS phenotype of small resistance arteries. Specifically, adult rats weaned on AIN-76A diet and switched to Teklad 8604 diet as adults had a loss of NOS-mediated vasorelaxation and NOS buffered PE-induced vasoconstriction in third-order mesenteric arteries. Interestingly, this loss of vascular NOS function was echoed by reduced expression of NOS3 as well as reduced expression of phosphorylated NOS3. In contrast, the group of Dahl S rats switched to AIN from the weaning Teklad diet demonstrated significantly enhanced phosphorylated NOS3 expression and maintenance of vascular NOS function. Vascular NOS function was intact in Dahl S rats weaned and maintained on Teklad or AIN standard diets with similar expression of phosphorylated NOS3.

Our laboratory is interested in determining the mechanisms that predispose salt-sensitive humans to vascular risk by utilizing the Dahl S rat model under normotensive (normal-salt diet) conditions. The major driving force behind the current study stems from the work in Dr. Mattson's laboratory where they reported that maintaining Dahl S rats at weaning on two different standard chow/normal salt diets differentially affected salt-dependent blood pressure and renal injury phenotypes as adults (27). Specifically, their study demonstrated that the response to a high-salt diet in adult Dahl S rats maintained on AIN-76A diet developed salt-dependent hypertension and greater renal injury compared to counterparts weaned on Teklad diet. We did not observe a difference in blood pressure between our Teklad diet and AIN-76A-diet groups or a difference in proteinuria. However, it is important to mention that the Teklad diet used in Mattson's study was Teklad 3075S diet; this diet was custom-made for the Mattson study (27). Whereas, our study exploited the widely available Teklad 8604 diet that many institutions, including our own, use as normal rat chow. Our laboratory has a long-
standing interest in examining vascular NOS function in cardiovascular disease states, thus prompting us to study arterial NOS function in Dahl S rats.

Using the non-specific NOS inhibitor, L-NAME, we showed a reduced sensitivity to ACh and increased sensitivity to PE in third-order mesenteric arteries from Teklad, AIN, and Teklad→AIN groups. These data indicate that NOS function is intact in these 3 diet groups; however, NOS function was lost in the AIN→Teklad group. Importantly, the vasorelaxation response to the exogenous NO-donor SNP was similar in all 4 diet groups, indicating the reduced NOS-mediated vasorelaxation in the AIN→Teklad group is not dependent on reduced vascular smooth muscle response to NO. In probing a mechanism for this loss in NOS function, it was observed that both total NOS3 and NOS3-phosphoserine1177 expression were reduced compared to AIN-fed rats. It has been demonstrated that reduced NOS3-p1177 expression is associated with reduced NOS3 enzyme activity (1). These data indicate that adaptation to the Teklad diet in adulthood from the AIN weaning diet results in dysfunctional vascular NOS signaling most likely due to reduced NOS3 and NOS3-p1177. Protein kinase B/Akt kinase phosphorylates NOS3 at ser-1177 to increase NOS3 activity (38). We propose that switching adult Dahl S rats to Teklad diet reduces small arterial Akt kinase function. Interestingly, the Teklad→AIN Dahl S rat group demonstrated reduced NOS3 expression whereas phosphorylated NOS3 was greatly enhanced suggesting that switching to AIN diet activates small arterial Akt kinase function. We probed the possible activation status of NOS in the Teklad→AIN by measuring metabolites of bioavailable NO; whereas plasma nitrite was similar in all diet groups, plasma nitrate was increased in the Teklad→AIN diet-switch group with no change in the AIN→Teklad diet-switch group. These data suggest that the Teklad→AIN diet switch but not the AIN→Teklad diet switch may influence bioavailable NO in Dahl S rats.
In adult Dahl S rats, basal ACh-mediated vasorelaxation and PE-mediated vasoconstriction in small mesenteric artery segments was not altered by placing rats on different standard chow diets (AIN or Teklad) at weaning or following a diet-switch protocol whereby the ‘weaning diets’ were switched as adults. Intriguingly, this was evident regardless of the loss of NOS function in the AIN→Teklad diet-switch group. Importantly, small artery vascular reactivity is modulated by NOS-independent mechanisms including endothelium-derived hyperpolarizing factors (EDHF) (8, 13, 40). Scotland et al demonstrated in small mesenteric arteries that EDHF functions to maintain normal vascular reactivity in NOS3 knockout mice (39). Future experiments will examine the degree of EDHF function in our Dahl S rat diet-switch groups.

Diet switch-induced changes in vascular NOS function and signaling in small arteries from adult Dahl S rats occurred without changes in blood pressure and heart rate or the circadian rhythm of these parameters; rat growth; or body weight. These data suggest that the changes in vascular NOS phenotype detected in our study are specific to the macronutrient composition and/or the source of each macronutrient in the Teklad versus AIN diet. Teklad 8604 is a proprietary diet containing 33% protein from soy, fish meal, wheat, corn, yeast, molasses and whey; 53% carbohydrates from corn, wheat, soy molasses, whey, and yeast; and 4% fat derived from soy, corn, wheat and fish. Whereas, AIN-76A is a purified diet composed of 19% protein derived from casein; 69% carbohydrates from corn starch and sucrose; and 12% fat from corn oil and trace amounts from casein. Currently, it is unknown which component in the diet is responsible for the observed change in vascular NOS phenotype; however we speculate that, although the protein in the Teklad diet comes from many sources, the total protein content is higher than in the AIN diet. In Dahl S rats, the consumption of a high-protein diet consisting of 33% protein and normal salt for 8 weeks induces greater vascular injury in the kidney compared to rats on a diet with normal (18%)
protein content (12); however, vascular NOS function or expression was not examined in that study. Our present investigation revealed that switching rats weaned on AIN diet, which has a normal protein composition compared to the Teklad diet, to the Teklad diet resulted in loss of NOS function and loss of NOS3 expression and signaling.

**Perspectives**

Collectively, our current study demonstrates that manipulating the standard chow/normal-salt diet in adult Dahl S rats, which mimic cardiovascular disease progression in salt sensitive humans, differentially affects small artery NOS phenotype. Although the Dahl S rat has been widely used to study mechanisms of high-salt diet-induced cardiovascular disease and NOS dysfunction (2, 4-7, 9, 10, 12, 14, 15, 18, 19, 21-23, 25, 27, 28, 30-37, 41, 46), far fewer studies have examined vascular NOS function or signaling in Dahl S rats maintained on normal-salt diet. Our current data suggests that switching standard chow diets alone may result in enhanced sensitivity to additional cardiovascular insults such as behavioral stressors or a high-salt diet in this rat strain. Our study should encourage investigators to more carefully consider that additional environmental stressors from dietary paradigms may influence the vascular NOS phenotype and therefore the vascular injury risk.
Acknowledgments
The authors greatly thank Hiram Ocasio for his technical expertise in biotelemetry. We greatly appreciate the efforts of Dr. Carol Moreno-Quinn at the Human and Molecular Genetics Center for genotyping our Dahl S rat breeders.

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Table 1. Body and tissue weights and metabolic parameters in Dahl S rats at 16 weeks old

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Teklad</th>
<th>Teklad→AIN</th>
<th>AIN</th>
<th>AIN→Teklad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibia Length (cm)</td>
<td>4.24±0.01 (7)</td>
<td>4.26±0.02 (9)</td>
<td>4.30±0.03 (6)</td>
<td>4.34±0.03 (8)</td>
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<tr>
<td>Body Weight (g/cm tibia)</td>
<td>100.21±1.76 (7)</td>
<td>98.04±2.35 (9)</td>
<td>96.12±1.28 (6)</td>
<td>97.84±2.55 (8)</td>
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<tr>
<td>Heart (g/cm tibia)</td>
<td>0.37±0.02 (7)</td>
<td>0.34±0.01 (9)</td>
<td>0.31±0.01 (6)</td>
<td>0.32±0.01 (8)</td>
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<td>Kidney (g/cm tibia)</td>
<td>0.81±0.03 (7)</td>
<td>0.73±0.01 (9)</td>
<td>0.71±0.03* (6)</td>
<td>0.75±0.03 (8)</td>
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<td>Epididymal Fat (g/cm tibia)</td>
<td>1.17±0.05 (7)</td>
<td>1.33±0.07 (9)</td>
<td>1.21±0.09 (6)</td>
<td>1.21±0.12 (8)</td>
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<tr>
<td>Food Intake (g/24h)</td>
<td>25.2±1.3 (8)</td>
<td>21.4±1.1 (9)</td>
<td>20.9±1.4 (7)</td>
<td>25.4±1.2 (5)</td>
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<tr>
<td>Water Intake (mL/24h)</td>
<td>30.1±1.8 (8)</td>
<td>21.8±1.6 (9)</td>
<td>18.4±2.4 (7)</td>
<td>30.1±0.8 (5)</td>
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<tr>
<td>Na Excretion (mEq/24h)</td>
<td>6.0±0.9 (7)</td>
<td>6.4±0.4 (9)</td>
<td>9.0±1.3 (7)</td>
<td>5.9±0.4 (4)</td>
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<td>Proteinuria (mg/24h)</td>
<td>0.6±0.1(6)</td>
<td>1.0±0.2(9)</td>
<td>0.7±0.07(6)</td>
<td>0.8±0.06(6)</td>
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Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 weeks old). At 12 weeks, weaning-diet groups were divided and a subset of rats underwent a diet switch generating two additional diet groups: Teklad→AIN and AIN→Teklad. Number of rats is in parentheses. *P<0.05 vs. Teklad; †P<0.05 vs. Analyzed by two-way ANOVA.
Table 2. Maximum response ($E_{\text{max}}$) and sensitivity ($\log E_{50}$) to acetylcholine (ACh) or sodium nitroprusside (SNP) in small mesenteric arteries from Dahl S rats at 16 weeks old

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<thead>
<tr>
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<tbody>
<tr>
<td><strong>$E_{\text{max}}$ to [ACh, 10$^{-5.5}$ M] and [SNP, 10$^{-6.5}$ M]</strong></td>
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<tr>
<td>ACh, % of PE</td>
<td>99.71±0.27(6)</td>
<td>99.53±0.41(10)</td>
<td>100.24±0.28(6)</td>
<td>100.14±0.37(6)</td>
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<td>ACh+L-NAME, % of PE</td>
<td>94.99±0.39(5)*</td>
<td>64.50±15.34(10)*</td>
<td>91.96±2.76(6)*</td>
<td>92.95±1.46(6)*</td>
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<td>SNP, % of PE</td>
<td>97.36±0.65(7)</td>
<td>96.65±1.91(10)</td>
<td>98.24±0.82(7)</td>
<td>98.54±0.51(6)</td>
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<td><strong>logEC$_{50}$</strong></td>
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<tr>
<td>ACh, [M]</td>
<td>-7.1±0.10(6)</td>
<td>-7.4±0.10(10)</td>
<td>-7.2±0.10(6)</td>
<td>-7.2±0.10(6)</td>
</tr>
<tr>
<td>ACh+L-NAME, [M]</td>
<td>-6.2±0.10(5)*</td>
<td>-6.3±0.20(8)*</td>
<td>-6.6±0.10(6)*</td>
<td>-6.7±0.20(5)</td>
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<td>SNP, [M]</td>
<td>-7.9±0.12(7)</td>
<td>-8.0±0.09(10)</td>
<td>-7.9±0.17(6)</td>
<td>-8.4±0.21(5)</td>
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Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 weeks old). At 12 weeks, weaning-diet groups were divided and a subset of rats underwent a diet switch generating two additional diet groups: Teklad→AIN and AIN→Teklad. L-NAME was used at [100$\mu$m] to non-selectively inhibit NOS. Data are mean±SEM. Number of rats is in parentheses. *$P<0.05$ vs. corresponding untreated mesenteric artery segment. Data analyzed by two-way ANOVA.
Table 3. Maximum response ($E_{\text{max}}$) and sensitivity ($\log EC_{50}$) to acetylcholine (ACh) or sodium nitroprusside (SNP) in aortae from Dahl S rats at 16 weeks old.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>$E_{\text{max}}$ to [ACh, 10^{-4.5} M] or [SNP, 10^{-5.5} M]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh, % of PE</td>
<td>70.84±4.18(4)</td>
<td>73.42±5.62(7)</td>
<td>80.04±2.63(4)</td>
<td>78.37±1.30(5)</td>
</tr>
<tr>
<td>ACh+L-NAME, % of PE</td>
<td>-19.03±4.01(6)*</td>
<td>-29.33±8.13(10)*</td>
<td>-12.94±4.38(7)*</td>
<td>-16.62±7.27(2)*</td>
</tr>
<tr>
<td>SNP, % of PE</td>
<td>98.76±0.68(6)</td>
<td>96.25±1.6(10)</td>
<td>95.47±2.53</td>
<td>99.39±0.74(6)</td>
</tr>
</tbody>
</table>

| **$\log EC_{50}$** |                 |                |                |                |
| ACh, [M]              | -6.31±0.23(4)   | -6.33±0.25(7)  | -6.49±0.13(4)  | -6.49±0.09(5)  |
| SNP, [M]              | -8.08±0.11(6)   | -7.93±0.15(10) | -8.37±0.13(7)  | -8.24±0.13(6)  |

Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 weeks old). At 12 weeks, weaning-diet groups were divided and a subset of rats underwent a diet switch generating two additional diet groups: Teklad→AIN and AIN→Teklad. L-NAME was used at [100μm] to non-selectively inhibit NOS. Number of rats is in parentheses. *P<0.05 vs. corresponding untreated aortic artery segment. Data analyzed by two-way ANOVA.
Table 4. Maximum response ($E_{max}$) and sensitivity (logEC$_{50}$) to phenylephrine (PE) or KCl in small mesenteric arteries from Dahl S rats at 16 weeks old

<table>
<thead>
<tr>
<th></th>
<th>Teklad</th>
<th>Teklad→AIN</th>
<th>AIN</th>
<th>AIN→Teklad</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$E_{max}$ to [PE, 10$^{-4.5}$ M] and [KCl, 100 mM]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE, % Increase in Force</td>
<td>694.94±34.53 (7)</td>
<td>607.58±51.55(10)</td>
<td>542.39±49.85(6)</td>
<td>641.52±62.37(6)</td>
</tr>
<tr>
<td>PE+L-NAME, % Increase in Force</td>
<td>756.89±61.56 (7)</td>
<td>626.89±61.39 (9)</td>
<td>564.27±57.82 (6)</td>
<td>665.33±79.91 (5)</td>
</tr>
<tr>
<td>KCl, % Increase in Force</td>
<td>312.35±20.21 (7)</td>
<td>301.44±27.24 (10)</td>
<td>204.02±27.88 (7)*</td>
<td>304.41±50.57 (6)</td>
</tr>
<tr>
<td><strong>logEC$_{50}$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE, [M]</td>
<td>-5.9±0.07 (7)</td>
<td>-5.9±0.06 (10)</td>
<td>-5.9±0.07 (6)</td>
<td>-5.9±0.1 (6)</td>
</tr>
<tr>
<td>PE+L-NAME, [M]</td>
<td>-6.1±0.02 (7)†</td>
<td>-6.2±0.09 (9)†</td>
<td>-6.3±0.1 (6)†</td>
<td>-6.0±0.08 (5)</td>
</tr>
<tr>
<td>KCl, [mM]</td>
<td>52.4±1.48 (7)</td>
<td>50.2±1.28 (10)</td>
<td>49.5±1.34 (7)</td>
<td>47.9±1.67 (6)</td>
</tr>
</tbody>
</table>

Dahl S rats were given **Teklad** or **AIN** standard chow diets at weaning (3 weeks old). At 12 weeks, weaning-diet groups were divided and a subset of rats underwent a diet switch generating two additional diet groups: **Teklad→AIN** and **AIN→Teklad**. L-NAME was used at [100μm] to non-selectively inhibit NOS. Data are mean±SEM. Number of rats is in parentheses. *$P<0.05$ for $E_{max}$ vs. Teklad; †$P<0.05$ vs. corresponding untreated mesenteric artery segment. Data analyzed by two-way ANOVA.
Table 5. Maximum response ($E_{\text{max}}$) and sensitivity (logEC$_{50}$) to phenylephrine (PE) or KCl in aortae from Dahl S rats at 16 weeks old

<table>
<thead>
<tr>
<th></th>
<th>Teklad</th>
<th>Teklad→AIN-76A</th>
<th>AIN-76A</th>
<th>AIN-76A→Teklad</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PE, % Increase in Force</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>137.93±14.39(6)</td>
<td>152.16±9.93(9)</td>
<td>127.51±11.16 ()</td>
<td>116.04±14.74(6)</td>
</tr>
<tr>
<td><strong>PE+L-NAME, % Increase in Force</strong></td>
<td>166.82±18.96(7)</td>
<td>172.94±8.77(9)</td>
<td>122.48±11.67(6)</td>
<td>130.79±11.69(6)</td>
</tr>
<tr>
<td><strong>KCl, % Increase in Force</strong></td>
<td>125.48±9.62(7)</td>
<td>123.82±3.13(10)</td>
<td>107.42±9.28(7)</td>
<td>108.88±14.28(5)</td>
</tr>
<tr>
<td><strong>logEC$_{50}$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE, [M]</td>
<td>-7.3±0.10(6)</td>
<td>-7.3±0.13(9)</td>
<td>-7.3±0.16(6)</td>
<td>-7.2±0.14(5)</td>
</tr>
<tr>
<td>PE+L-NAME, [M]</td>
<td>-7.6±0.16(7)</td>
<td>-7.6±0.09(9)</td>
<td>-7.5±0.15(6)</td>
<td>-7.3±0.15(5)</td>
</tr>
<tr>
<td>KCl, [mM]</td>
<td>24.6±3.21(7)±</td>
<td>25.0±0.75(10)</td>
<td>25.1±2.27(7)</td>
<td>22.9±4.34(4)</td>
</tr>
</tbody>
</table>

Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 weeks old). At 12 weeks, weaning-diet groups were divided and a subset of rats underwent a diet switch generating two additional diet groups: Teklad→AIN and AIN→Teklad. L-NAME was used at [100μm] to non-selectively inhibit NOS. Number of rats is in parentheses. Data analyzed by two-way ANOVA.
Figure Legends

Figure 1. A: Diagram of diet-switch protocol performed in Dahl S rats. Rats were fed Teklad diet or AIN diet at weaning (3 weeks old). Rats either remained on respective diet until 16 weeks old or, at 12 weeks old, diets were switched with Teklad-fed rats changed to AIN diet (Teklad→AIN) or AIN-fed rats changed to Teklad diet (AIN→Teklad). B: Weekly body weights for Dahl S rats for Teklad (N=9), AIN (N=9), Teklad→AIN (N=10), and AIN→Teklad (N=9). Data analyzed by two-way ANOVA.

Figure 2. 24h (A) mean arterial blood pressure (MAP) (A) and heart rate (B) tracings in Dahl S rats fed Teklad (N=6) or AIN (N=4) standard chow diets since weaning (3 weeks old); 12h MAP (C) and heart rate (D) (at 15 weeks, 5 and 6 days old and at 16 weeks old). At 12 weeks old, weaning-diet groups were divided with a subset of rats undergoing a diet switch thereby generating two additional diet groups: Teklad→AIN (N=6) and AIN→Teklad (N=3). Data analyzed by two-way ANOVA.

Figure 3. Acetylcholine (ACh)-mediated relaxation in the presence or absence of L-NAME in small mesenteric arteries from adult (16 weeks old) Dahl S rats fed Teklad (A; N=6) or AIN (B; N=6) since weaning (3 weeks old). At 12 weeks old, weaning-diet groups were divided with a subset of rats undergoing a change in diet generating two additional diet groups: Teklad→AIN (C; N=10) and AIN→Teklad (D; N=6). *P<0.05 for logEC_{50} of L-NAME-treated vs. untreated mesenteric artery segments. Data analyzed by t-test.

Figure 4. Phenylephrine (PE)-induced relaxation in the presence or absence of L-NAME in small mesenteric arteries from adult (16 weeks old) Dahl S rats fed Teklad (A; N=7) or AIN (B; N=6) since weaning (3 weeks old). At 12 weeks old, weaning-diet groups were divided with a
subset of rats undergoing a diet switch generating two additional diet groups: Teklad→AIN (C; N=9) and AIN→Teklad (D; N=5). *P<0.05 for logEC50 in L-NAME-treated vs. untreated mesenteric artery segments. Data analyzed by t-test.

Figure 5. Densitometric analysis and representative Western blots of NOS3/β-actin (A, B), NOS3-p1177/NOS3 (C, D) protein expression in homogenates of mesenteric arteries and plasma nitrite (E) and nitrate (F) levels from Teklad-fed (T; N=5) and AIN-fed (A; N=4) adult (16 weeks old) Dahl S rats. At 12 weeks old, weaning-diet groups were divided with a subset of rats undergoing a diet switch thereby generating two additional diet groups: Teklad→AIN (T→A; N=7) and AIN→Teklad (A→T; N=6). *P<0.05 vs. T; †P<0.05 vs. corresponding weaning group (T or A). Data analyzed by two-way ANOVA.


44. Weinberger MH. Salt sensitivity is associated with an increased mortality in both normal and hypertensive humans. *Journal of clinical hypertension (Greenwich, Conn)* 4: 274-276, 2002.


Figure 1

A) Diet-switch protocol

Standard chow diets provided at 3 weeks old:
- Teklad 8604
- AIN-76A

Standard chow diets provided at 12 weeks old:
- Teklad 8604
- AIN-76A

Nomenclature for experimental groups at 16 weeks old:
- Teklad
- Teklad → AIN
- AIN
- AIN → Teklad

B) Body Weight (g) vs. Age (Weeks)

DIET SWITCH

- □ Teklad
- □ Teklad → AIN
- ▪ AIN
- ▲ AIN → Teklad
Figure 2
Figure 3

A. Relaxation (% of PE) vs. -log [ACh, M] for groups Teklad and Teklad+L-NAME.

B. Relaxation (% of PE) vs. -log [ACh, M] for groups AIN and AIN+L-NAME.

C. Relaxation (% of PE) vs. -log [ACh, M] for groups Teklad→AIN and Teklad→AIN+L-NAME.

D. Relaxation (% of PE) vs. -log [ACh, M] for groups AIN→Teklad and AIN→Teklad+L-NAME.
Figure 4

A

% Increase in Force

-log [PE, M]

B

% Increase in Force

-log [PE, M]

C

% Increase in Force

-log [PE, M]

D

% Increase in Force

-log [PE, M]
Figure 5

A

![Graph A](image)

B

![Image B](image)

C

![Graph C](image)

D

![Image D](image)

E

![Graph E](image)

F

![Graph F](image)