

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

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Spradley FT, Ho DH, Kang KT, Pollock DM, Pollock JS. Changing standard chow diet promotes vascular NOS dysfunction in Dahl S rats. *Am J Physiol Regul Integr Comp Physiol* 302: R150–R158, 2012. First published October 26, 2011; doi:10.1152/ajpregu.00482.2011.—We hypothesized that vascular nitric oxide synthase (NOS) function and expression is differentially regulated in adult Dahl salt-sensitive rats maintained on Teklad or American Institutes of Nutrition (AIN)-76A standard chow diets from 3 to 16 wk old. At 16 wk old, acetylcholine (ACh)-mediated vasorelaxation and phenylephrine (PE)-mediated vasoconstriction in the presence and absence of NOS inhibitor, *N*^ω-nitro-L-arginine methyl ester (L-NAME), was assessed in small-resistance mesenteric arteries and aortas. 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 salt-sensitive rats to vascular disease.

standard diet; Dahl rat; *N*^ω-nitro-L-arginine methyl ester; nitric oxide synthase; vascular reactivity

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; α_1 -specific agonist) compared with 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 wk old. Mesenteric arterial and aortic reactivity, as well as NOS function and expression were assessed at 16 wk old.

MATERIALS AND METHODS

Animal model. Dahl S rat breeders were purchased from Charles River Laboratories (Wilmington, MA) and placed on Teklad 8604 diet on arrival at Georgia Health Sciences University (GHSU). First-generation Dahl S rats from six breeding pairs were used in this study. A genomewide 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 wk 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

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contained 0.4% NaCl with similar vitamin compositions. All rats were given tap water ad libitum.

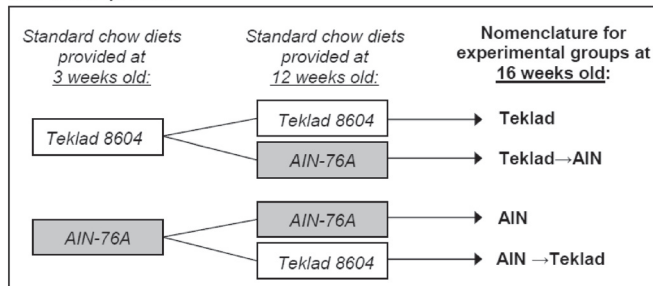
At 12 wk old, Teklad-weaned or AIN-weaned rats underwent a diet-switch protocol, where a subset of Teklad rats was switched to AIN and vice versa. Importantly, rats generated from each breeding pair were included in each of the four diet groups (Fig. 1A). Weekly body weights were assessed on all rats. At 16 wk 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 3,000 g for 10 min, and snap-frozen in liquid N₂. Mesenteric arteries and aortas 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 wk old, as described previously (16). Rats recovered from surgery for ~1 wk, while having free access to tap water and their respective diet. From 12–16 wk old, the diet-switch protocol was performed (Fig. 1A), while mean arterial blood pressure measurements were collected every 10th min. Blood pressure is reported as a 24-h average or 12-h average.

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

A

Diet-switch protocol



B

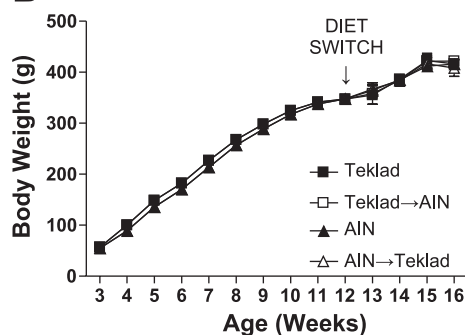


Fig. 1. A: diagram of diet-switch protocol performed in Dahl salt-sensitive (S) rats. Rats were fed Teklad diet or American Institutes of Nutrition (AIN) diet at weaning (3 wk old). Rats either remained on respective diet until 16 wk old, or, at 12 wk old, diets were switched, with Teklad-fed rats changed 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 were analyzed by two-way ANOVA.

Vascular reactivity. Thoracic aortas and third-order mesenteric arteries were cleaned of adherent fat, 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 μ M and 2 μ M PE, respectively, followed by evaluation of vasorelaxation with cumulative-concentration response curves to acetylcholine (ACh; 1×10^{-9} M to $3 \times 10^{-5.5}$ M for mesenteric arteries and 1×10^{-9} M to $3 \times 10^{-4.5}$ M for aortas) and then to sodium nitroprusside (SNP; 1×10^{-10} M to $3 \times 10^{-6.5}$ M for mesenteric arteries and 1×10^{-10} M to $3 \times 10^{-5.5}$ M for aortas) in the same artery segment. Vasorelaxation data are presented as relaxation (%PE constriction), as analyzed by the equation [(maximum PE response – ACh response)/(maximum PE response – baseline before PE constriction)] \times 100. Vasoconstriction was assessed with PE (1×10^{-9} M to 3×10^{-5} M), followed by KCl concentration-response curve (8×10^{-3} M to 100×10^{-3} M). Constriction responses are presented as percent increase in force, as analyzed by the equation [(response to vasoconstrictor – baseline before constriction)/baseline before constriction] \times 100. Rings were incubated in the presence or absence of the nonspecific NOS inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME; 100 μ M; Sigma) for 15 min before construction of response curves. Maximum response and sensitivity to the vasoactive agonists are expressed as E_{max} and logEC₅₀, respectively. LogEC₅₀ 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 400- μ l ice-cold lysis buffer (50 mM Tris, 0.1 mM EDTA disodium salt, 0.1 mM EGTA, 0.1 mM sucrose, 0.1% 2-mercaptoethanol, 10% glycerol, 2 μ M leupeptin, 2 μ M pepstatin A, 1 mM 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 g at 4°C for 5 min, and supernatants were isolated. Protein concentrations of supernatants were determined (BCA assay, Bio-Rad). Thirty micrograms of protein were separated via 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed using anti-NOS1, anti-NOS3, anti-NOS3-phosphoserine-1177 (p1177; 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 (1:1,000; Invitrogen, Carlsbad, CA), and β -actin was 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-p1177 was normalized to NOS3 expression.

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

Statistical analyses. All data are expressed as means \pm SE. 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 wk until 16 wk old, gained weight similarly and had comparable tibia lengths, demonstrating that neither standard diet differentially affected rat growth (Fig. 1B; Table 1). Food and water intakes were also similar at 16 wk 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

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

Parameter	Teklad	Teklad→AIN	AIN	AIN→Teklad
Tibia length, cm	4.24 ± 0.01 (7)	4.26 ± 0.02 (9)	4.30 ± 0.03 (6)	4.34 ± 0.03 (8)
Body weight, g/cm tibia	100.21 ± 1.76 (7)	98.04 ± 2.35 (9)	96.12 ± 1.28 (6)	97.84 ± 2.55 (8)
Heart, g/cm tibia	0.37 ± 0.02 (7)	0.34 ± 0.01 (9)	0.31 ± 0.01 (6)	0.32 ± 0.01 (8)
Kidney, g/cm tibia	0.81 ± 0.03 (7)	0.73 ± 0.01 (9)	0.71 ± 0.03* (6)	0.75 ± 0.03 (8)
Epididymal fat, g/cm tibia	1.17 ± 0.05 (7)	1.33 ± 0.07 (9)	1.21 ± 0.09 (6)	1.21 ± 0.12 (8)
Food intake, g/24 h	25.2 ± 1.3 (8)	21.4 ± 1.1 (9)	20.9 ± 1.4 (7)	25.4 ± 1.2 (5)
Water intake, ml/24 h	30.1 ± 1.8 (8)	21.8 ± 1.6 (9)	18.4 ± 2.4 (7)	30.1 ± 0.8 (5)
Na excretion, meq/24 h	6.0 ± 0.9 (7)	6.4 ± 0.4 (9)	9.0 ± 1.3 (7)	5.9 ± 0.4 (4)
Proteinuria, mg/24 h	0.6 ± 0.1 (6)	1.0 ± 0.2 (9)	0.7 ± 0.07 (6)	0.8 ± 0.06 (6)

Values are means ± SE; no. of rats are in parentheses. Dahl salt-sensitive (S) rats were given Teklad or American Institutes of Nutrition (AIN) standard chow diets at weaning (3 wk old). At 12 wk, weaning-diet groups were divided, and a subset of rats underwent a diet switch, generating two additional diet groups: Teklad→AIN and AIN→Teklad. * $P < 0.05$ vs. Teklad. Data were analyzed by two-way ANOVA.

groups raised on the AIN diet vs. the Teklad diet, with no difference between the other groups (Table 1).

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

Hemodynamic and activity measurements. At 16 wk old, 24-h mean arterial pressure (MAP) and heart rate were similar in the nonswitched weaning diet groups (Teklad or AIN); the trend for increased 24-h MAP in the diet-switch groups (Teklad→AIN or AIN→Teklad) was not significant (Fig. 2A). Additionally, the tendency for 24-h MAP to increase from 12 to 16 wk old was not statistically different between the four diet groups (Fig. 2A). At 16 wk old, 24-h heart rate was similar

in all diet groups (Fig. 2B). Circadian rhythms of 12-h MAP (Fig. 2C) and heart rate (Fig. 2D) were similar in all groups during the last 3 days of the diet-switch protocol (at 15 wk, 5 and 6 days old and at 16 wk old).

Vasorelaxation. Cumulative concentration-response curves to ACh were generated to assess endothelial function in third-order small-resistance mesenteric arteries and thoracic aortas. No difference in maximum relaxation (E_{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 nitric oxide (NO) donor, SNP, between all four groups of Dahl S rats (Table 2).

To assess NOS function, ACh-mediated relaxation curves were generated in the presence of the nonspecific NOS inhibitor, L-NAME. L-NAME significantly reduced sensitivity to ACh in Teklad (Fig. 3A; Table 2), AIN (Fig. 3B; Table 2), and Teklad→AIN (Fig. 3C; Table 2) diet groups; however, the trend for L-NAME pretreatment to reduce ACh sensitivity in

Fig. 2. Twenty-four-hour 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 wk old). Twelve-hour MAP (C) and heart rate (D) (at 15 wk, 5 and 6 days old, and at 16 wk old) are shown. At 12 wk 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 ($N = 3$). Values are means ± SE. Data were analyzed by two-way ANOVA. N, night; D, day; bpm, beats per minute.

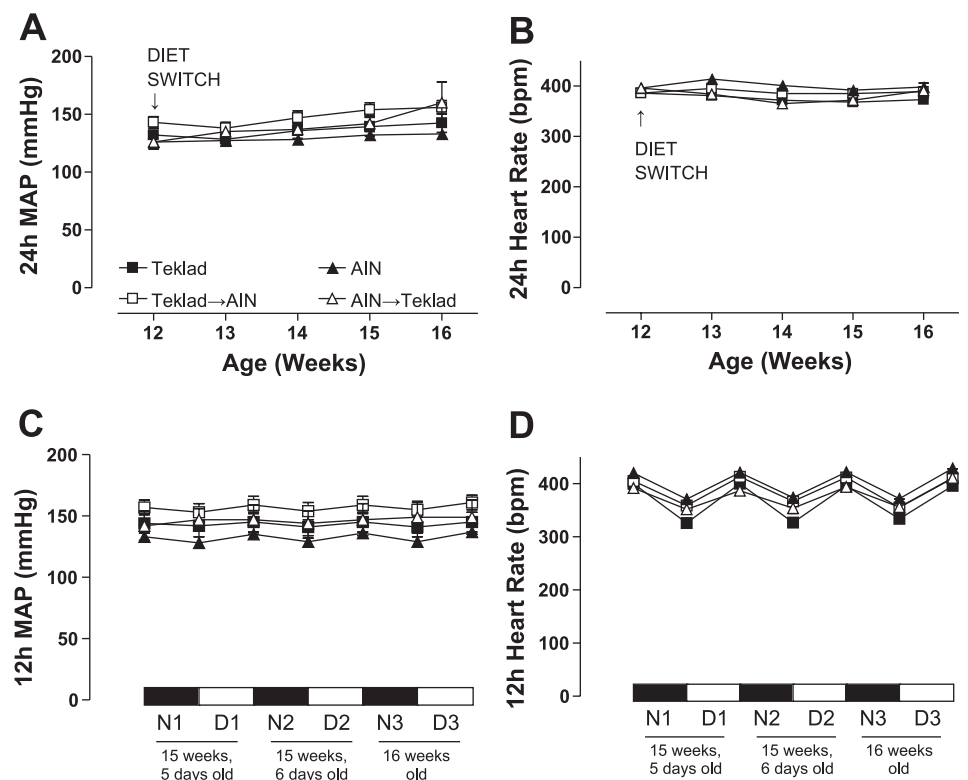


Table 2. Maximum response (E_{max}) and sensitivity ($\log EC_{50}$) to ACh or SNP in small mesenteric arteries from Dahl S rats at 16 wk old

	Teklad	Teklad→AIN	AIN	AIN→Teklad
E_{max} to [ACh, $10^{-5.5}$ M] and [SNP, $10^{-6.5}$ M]				
ACh, %PE	99.71 ± 0.27 (6)	99.53 ± 0.41 (10)	100.24 ± 0.28 (6)	100.14 ± 0.37 (6)
ACh + L-NAME, %PE	94.99 ± 0.39* (5)	64.50 ± 15.34* (10)	91.96 ± 2.76* (6)	92.95 ± 1.46* (6)
SNP, %PE	97.36 ± 0.65 (7)	96.65 ± 1.91 (10)	98.24 ± 0.82 (7)	98.54 ± 0.51 (6)
$\log EC_{50}$				
ACh, M concn.	-7.1 ± 0.10 (6)	-7.4 ± 0.10 (10)	-7.2 ± 0.10 (6)	-7.2 ± 0.10 (6)
ACh + L-NAME, M concn.	-6.2 ± 0.10* (5)	-6.3 ± 0.20* (8)	-6.6 ± 0.10* (6)	-6.7 ± 0.20 (5)
SNP, M concn.	-7.9 ± 0.12 (7)	-8.0 ± 0.09 (10)	-7.9 ± 0.17 (6)	-8.4 ± 0.21 (5)

Values are means ± SE; no. of rats are in parentheses. Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 wk old). At 12 wk, weaning-diet groups were divided, and a subset of rats underwent a diet switch, generating two additional diet groups: Teklad→AIN and AIN→Teklad. *N*^o-nitro-L-arginine methyl ester (L-NAME) was used at 100- μ M concentration to nonselectively inhibit nitric oxide synthase (NOS). ACh, acetylcholine; SNP, sodium nitroprusside; PE, phenylephrine. Brackets denote concentration. * P < 0.05 vs. corresponding untreated mesenteric artery segment. Data were analyzed by two-way ANOVA.

mesenteric artery segments from AIN→Teklad Dahl S rats was not statistically significant (Fig. 3D; Table 2).

Aortic vasorelaxation to ACh and SNP was similar in all four 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 four 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 vs. all other diet groups (Table 4).

L-NAME pretreatment significantly increased sensitivity to PE-induced constriction in mesenteric arteries isolated from

Teklad-fed (Fig. 4A; Table 4), AIN-fed (Fig. 4B; Table 4), and Teklad→AIN (Fig. 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 (Fig. 4D; Table 4).

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

NOS protein expression. NOS3 protein expression was significantly reduced in small mesenteric arteries from AIN and Teklad→AIN compared with Teklad (Fig. 5, A and B). Moreover, NOS3 protein expression was further reduced in small mesenteric arteries from AIN→Teklad compared with AIN (Fig. 5, A and B). The NOS3-p1177 expression (Fig. 5, C and D) was similar in small mesenteric arteries from Teklad and

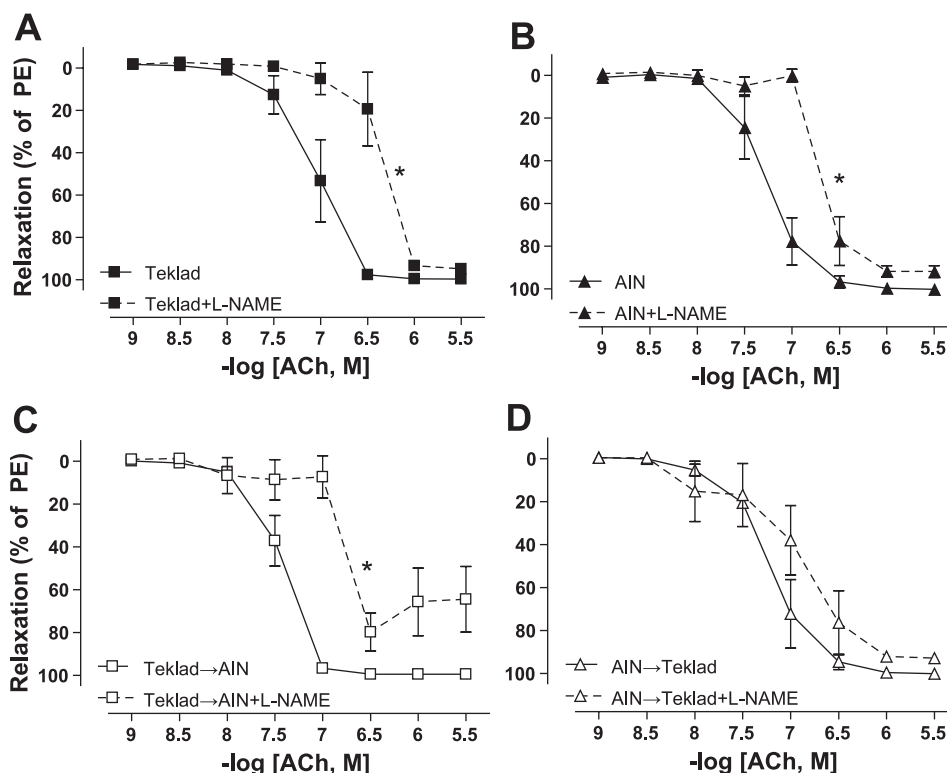


Fig. 3. Acetylcholine (ACh)-mediated relaxation in the presence or absence of *N*^o-nitro-L-arginine methyl ester (L-NAME) in small mesenteric arteries from adult (16 wk old) Dahl S rats fed Teklad ($N = 6$; A) or AIN ($N = 6$; B) since weaning (3 wk old). At 12 wk old, weaning-diet groups were divided, and a subset of rats underwent a diet switch, generating two additional diet groups: Teklad→AIN ($N = 10$; C) and AIN→Teklad ($N = 6$; D). *N*^o-nitro-L-arginine methyl ester (L-NAME) was used at 100 μ M to nonselectively inhibit nitric oxide synthase (NOS). Values are means ± SE. * P < 0.05 for maximum sensitivity to the vasoactive agonists ($\log EC_{50}$) of L-NAME-treated vs. untreated mesenteric artery segments. Data were analyzed by *t*-test. PE, phenylephrine.

Table 3. Maximum response (E_{max}) and sensitivity ($\log EC_{50}$) to ACh or SNP in aortas from Dahl S rats at 16 wk old

	Teklad	Teklad→AIN	AIN	AIN→Teklad
E_{max} to [ACh, $10^{-4.5}$ M] or [SNP, $10^{-5.5}$ M]				
ACh, %PE	70.84 ± 4.18 (4)	73.42 ± 5.62 (7)	80.04 ± 2.63 (4)	78.37 ± 1.30 (5)
ACh + L-NAME, %PE	-19.03 ± 4.01* (6)	-29.33 ± 8.13* (10)	-12.94 ± 4.38* (7)	-16.62 ± 7.27* (6)
SNP, %PE	98.76 ± 0.68 (6)	96.25 ± 1.6 (10)	95.47 ± 2.53	99.39 ± 0.74 (6)
$\log EC_{50}$				
ACh, M concn.	-6.31 ± 0.23 (4)	-6.33 ± 0.25 (7)	-6.49 ± 0.13 (4)	-6.49 ± 0.09 (5)
SNP, M concn.	-8.08 ± 0.11 (6)	-7.93 ± 0.15 (10)	-8.37 ± 0.13 (7)	-8.24 ± 0.13 (6)

Values are means ± SE; no. of rats are in parentheses. Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 wk old). At 12 wk, 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- μ M concentration to nonselectively inhibit NOS. * P < 0.05 vs. corresponding untreated aortic artery segment. Data were analyzed by two-way ANOVA.

AIN. NOS3-p1177 expression in arteries from Teklad→AIN was significantly higher compared with arteries from Teklad (Fig. 5, C and D), while the arteries from AIN→Teklad demonstrated a significantly reduced NOS3-p1177 expression compared with AIN (Fig. 5, C and D). NOS1 expression was not significantly different between the four diet groups (data not shown).

Plasma nitrite/nitrate. Nitrite levels were similar in all four diet groups (Fig. 5E). Moreover, nitrate levels were similar in nonswitched weaning diet groups (Fig. 5F). Interestingly, nitrate levels were increased following the Teklad→AIN diet switch, whereas no change was detected in the AIN→Teklad diet-switch group (Fig. 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 present 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 with 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 (27) was Teklad 3075S diet; this diet was custom-made for the Mattson study, 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 nonspecific 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

Table 4. Maximum response (E_{max}) and sensitivity ($\log EC_{50}$) to PE or KCl in small mesenteric arteries from Dahl S rats at 16 wk old

	Teklad	Teklad→AIN	AIN	AIN→Teklad
E_{max} to [PE, $10^{-4.5}$ M] and [KCl, 100 mM]				
PE, %increase in force	694.94 ± 34.53 (7)	607.58 ± 51.55 (10)	542.39 ± 49.85 (6)	641.52 ± 62.37 (6)
PE + L-NAME, %increase in force	756.89 ± 61.56 (7)	626.89 ± 61.39 (9)	564.27 ± 57.82 (6)	665.33 ± 79.91 (5)
KCl, %increase in force	312.35 ± 20.21(7)	301.44 ± 27.24 (10)	204.02 ± 27.88* (7)	304.41 ± 50.57 (6)
$\log EC_{50}$				
PE, M concn.	-5.9 ± 0.07 (7)	-5.9 ± 0.06 (10)	-5.9 ± 0.07 (6)	-5.9 ± 0.1 (6)
PE + L-NAME, M concn.	-6.1 ± 0.02† (7)	-6.2 ± 0.09† (9)	-6.3 ± 0.1† (6)	-6.0 ± 0.08 (5)
KCl, mM concn.	52.4 ± 1.48 (7)	50.2 ± 1.28 (10)	49.5 ± 1.34 (7)	47.9 ± 1.67 (6)

Values are means ± SE; no. of rats are in parentheses. Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 wk old). At 12 wk, 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- μ M concentration to nonselectively inhibit NOS. * P < 0.05 for E_{max} vs. Teklad. † P < 0.05 vs. corresponding untreated mesenteric artery segment. Data were analyzed by two-way ANOVA.

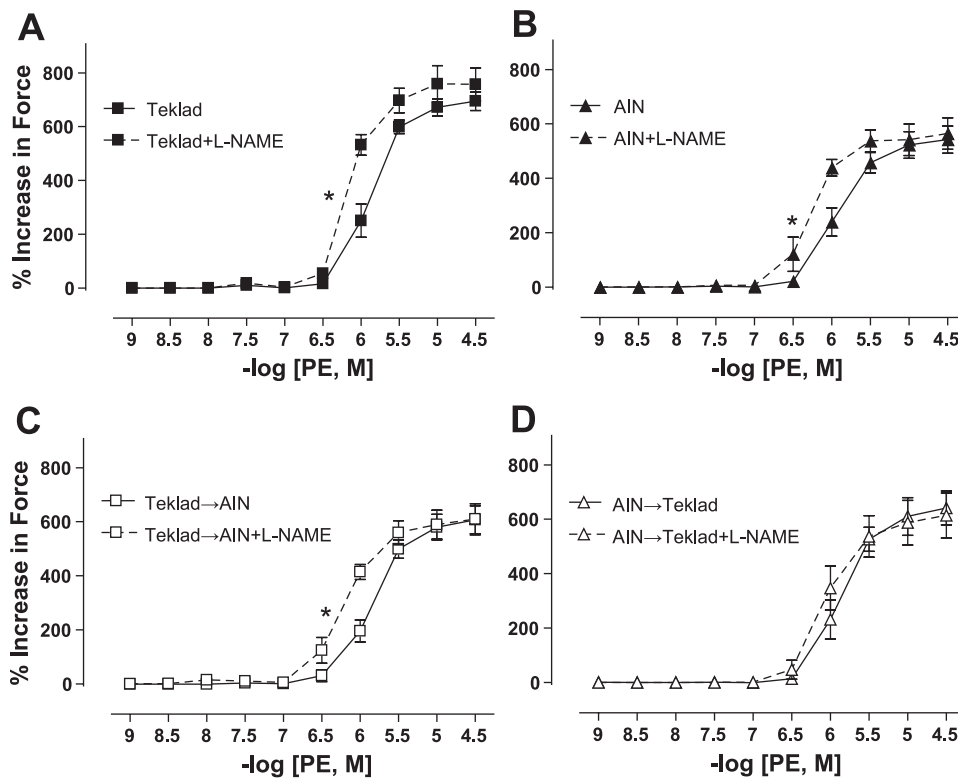


Fig. 4. Phenylephrine (PE)-induced concentration in the presence or absence of L-NAME in small mesenteric arteries from adult (16 wk old) Dahl S rats fed Teklad ($N = 7$; A) or AIN ($N = 6$; B) since weaning (3 wk old). At 12 wk old, weaning-diet groups were divided, with a subset of rats undergoing a diet switch, generating two additional diet groups: Teklad→AIN ($N = 9$; C) and AIN→Teklad ($N = 5$; D). L-NAME was used at 100 μM concentration to nonselectively inhibit NOS. Values are means \pm SE. * $P < 0.05$ for $\log\text{EC}_{50}$ in L-NAME-treated vs. untreated mesenteric artery segments. Data were analyzed by *t*-test.

NOS function is intact in these three 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 four 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-p1177 expression were reduced compared with 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 Ser1177 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

Table 5. Maximum response (E_{max}) and sensitivity ($\log\text{EC}_{50}$) to PE or KCl in aortas from Dahl S rats at 16 wk old

	Teklad	Teklad→AIN	AIN	AIN→Teklad
E_{max} to [PE, $10^{-4.5}$ M] or [KCl, 100 mM]				
PE, %increase in force	137.93 \pm 14.39 (6)	152.16 \pm 9.93 (9)	127.51 \pm 11.16 (6)	116.04 \pm 14.74 (6)
PE + L-NAME, %increase in force	166.82 \pm 18.96 (7)	172.94 \pm 8.77 (9)	122.48 \pm 11.67 (6)	130.79 \pm 11.69 (6)
KCl, %increase in force	125.48 \pm 9.62 (7)	123.82 \pm 3.13 (10)	107.42 \pm 9.28 (7)	108.88 \pm 14.28 (5)
$\log\text{EC}_{50}$				
PE, M concn.	-7.3 \pm 0.10 (6)	-7.3 \pm 0.13 (9)	-7.3 \pm 0.16 (6)	-7.2 \pm 0.14 (5)
PE + L-NAME, M concn.	-7.6 \pm 0.16 (7)	-7.6 \pm 0.09 (9)	-7.5 \pm 0.15 (6)	-7.3 \pm 0.15 (5)
KCl, mM concn.	24.6 \pm 3.21 (7)	25.0 \pm 0.75 (10)	25.1 \pm 2.27 (7)	22.9 \pm 4.34 (4)

Values are means \pm SE; no. of rats are in parentheses. Dahl S rats were given Teklad or AIN standard chow diets at weaning (3 wk old). At 12 wk, 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- μM concentration to nonselectively inhibit NOS. Data were analyzed by two-way ANOVA.

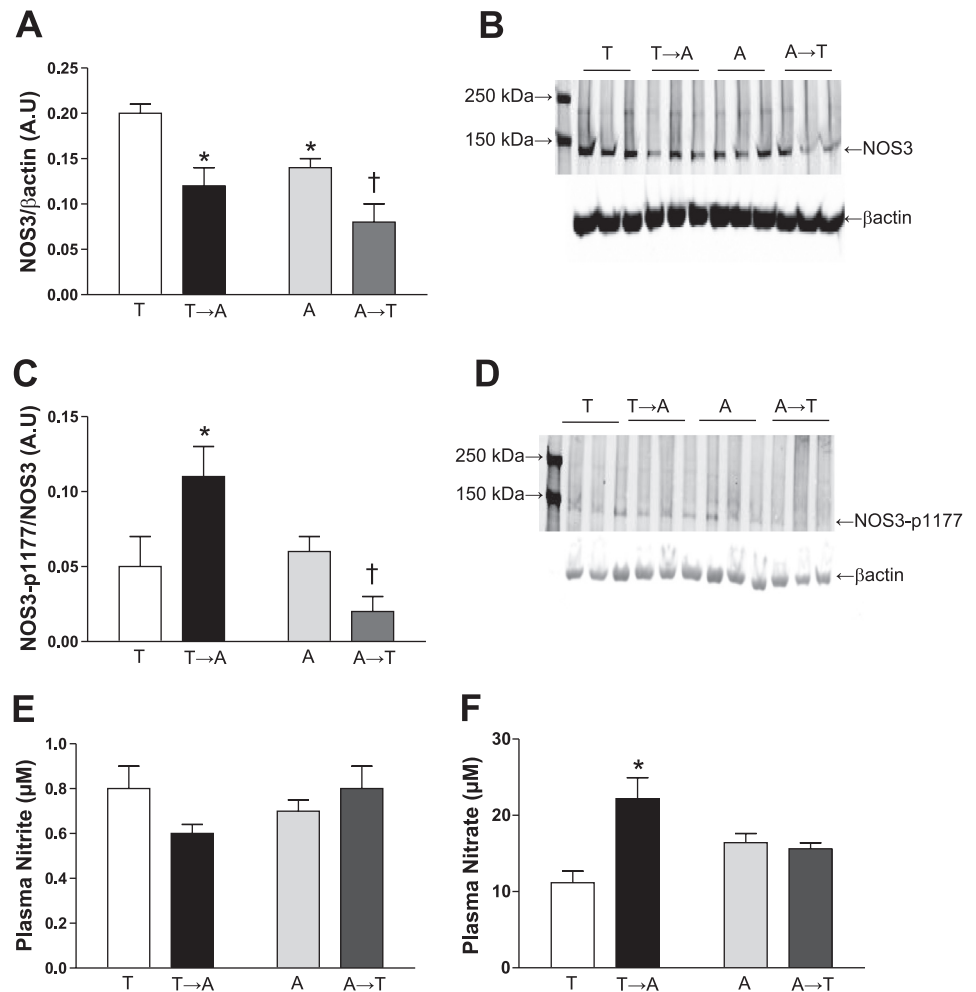


Fig. 5. Densitometric analysis and representative Western blots of nitric oxide synthase (NOS) 3/ β -actin (A and B), NOS3-phosphoserine-1177 (p1177)/NOS3 (C and D) protein expression in homogenates of mesenteric arteries and plasma nitrite (E) and nitrate (F) levels from Teklad-fed (T; $N = 5$) and AIN-fed (A; $N = 4$) adult (16-wk-old) Dahl S rats. At 12 wk old, weaning-diet groups were divided, with a subset of rats undergoing a diet switch, thereby generating two additional diet groups: Teklad \rightarrow AIN (T \rightarrow A; $N = 7$) and AIN \rightarrow Teklad (A \rightarrow T; $N = 6$). Values are means \pm SE. * $P < 0.05$ vs. T. † $P < 0.05$ vs. corresponding weaning group (T or A). Data were analyzed by two-way ANOVA. AU, arbitrary units.

NOS function in the AIN \rightarrow 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. (39) demonstrated in small mesenteric arteries that EDHF functions to maintain normal vascular reactivity in NOS3 knockout mice. 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 vs. 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 14% 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 that in the AIN diet. In Dahl S rats, the consumption of a high-protein diet consisting of 33% protein and normal salt for 8 wk induces greater vascular injury in the kidney compared with 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 with the Teklad diet, to the Teklad diet resulted in loss of NOS function and loss of NOS3 expression and signaling.

Perspectives

Collectively, our present 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 present data suggest 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.T.S. and J.S.P. conception and design of research; F.T.S., D.H.H., and K.-T.K. performed experiments; F.T.S., D.H.H., K.-T.K., and J.S.P. analyzed data; F.T.S., D.H.H., D.M.P., and J.S.P. interpreted results of experiments; F.T.S. prepared figures; F.T.S. drafted manuscript; F.T.S., D.H.H., K.-T.K., D.M.P., and J.S.P. edited and revised manuscript; F.T.S., D.H.H., K.-T.K., D.M.P., and J.S.P. approved final version of manuscript.

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