Excessive dietary salt promotes aortic stiffness in murine renovascular hypertension

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DeLalio LJ, Hahn S, Katayama PL, Wenner MM, Farquhar WB, Straub AC, Stocker SD. Excessive dietary salt promotes aortic stiffness in murine renovascular hypertension. Am J Physiol Heart Circ Physiol 318: H1346–H1355, 2020. First published April 17, 2020; doi:10.1152/ajpheart.00601.2019.—Renovascular hypertension is characterized by activation of the renin-angiotensin-aldosterone system, blunted natriuretic responses, and elevated sympathetic nerve activity. Excess dietary salt intake exaggerates arterial blood pressure (ABP) in multiple models of experimental hypertension. The present study tested whether a high-salt diet exaggerated ABP and vascular dysfunction in a 2-kidney, 1-clip (2K1C) murine model. Male C57BL/6J mice (8–12 wk) were randomly assigned, and fed a 0.1% or 4.0% NaCl diet, and instrumented with telemetry units to measure ABP. Then, the 2K1C model was produced by placing a cuff around the right renal artery. Systolic, diastolic, and mean ABP were significantly higher in mice fed 4.0% vs. 0.1% NaCl at 1 wk but not after 3 wk. Interestingly, 2K1C hypertension progressively increased arterial pulse pressure in both groups; however, the magnitude was significantly greater in mice fed 4.0% vs. 0.1% NaCl at 1 wk but not after 3 wk. Moreover, pulse wave velocity was significantly greater in 2K1C mice fed 4.0% vs. 0.1% NaCl diet or sham-operated mice fed either diet. Histological assessment of aortas indicated no structural differences among groups. Finally, endothelium-dependent vasodilation was significantly and selectively attenuated in the aorta but not mesenteric arteries of 2K1C mice fed 4.0% NaCl diet vs. 0.1% NaCl or sham-operated control mice. The findings suggest that dietary salt loading transiently exaggerates 2K1C renovascular hypertension but promotes chronic aortic stiffness and selective aortic vascular dysfunction.

NEW & NOTEWORTHY High dietary salt exaggerates hypertension in multiple experimental models. Here we demonstrate that a high-salt diet produces a greater increase in arterial blood pressure at 1 wk after induction of 2-kidney, 1-clip (2K1C) hypertension but not at 3 wk. Interestingly, 2K1C mice fed a high-salt diet displayed an exaggerated pulse pressure, elevated pulse wave velocity, and reduced endothelium-dependent vasodilation of the aorta but not mesenteric arteries. These findings suggest that dietary salt may interact with underlying cardiovascular disease to promote selective vascular dysfunction and aortic stiffness.

INTRODUCTION Renovascular hypertension is a common form of secondary hypertension that accounts for 2–5% of systemic hypertension and 24.0% of resistant hypertension (3, 9). Commonly, atherosclerotic lesions in one or both renal arteries engender renal hypoperfusion and ischemia (60). The resultant rise in arterial blood pressure (ABP) has been attributed to multiple factors including activation of the renin-angiotensin-aldosterone system (RAAS), renal dysfunction and blunted natriuresis, and elevated sympathetic outflow. The relative contribution of each mechanism may differ over time. For example, the early phase of 2-kidney, 1-clip (2K1C) hypertension is characterized by a robust activation of the RAAS (6, 49), and pharmacological or genetic inhibition of RAAS attenuates hypertension in 2K1C models (7, 36, 48). As 2K1C hypertension persists, a neurogenic-mediated increase in total peripheral resistance due to elevated sympathetic outflow also contributes to the elevated ABP (11, 24, 42). The mechanisms underlying the sympathoexcitation are unknown but attributed to the central action of systemic angiotensin II and activation of renal sensory nerves (32, 41).

Excess dietary salt intake is strongly correlated with cardiovascular disease and is regarded as a major contributing factor to the pathogenesis of hypertension (1, 61, 62). Multiple systems contribute to salt-sensitive hypertension including impaired renal and vascular function and the sympathetic nervous system (30, 43, 44, 55, 57). Experimentally, dietary salt loading has been repeatedly demonstrated to increase and exaggerate ABP in multiple experimental models including Dahl salt-sensitive rats (22, 38), deoxycorticosterone acetate-salt (23, 25), chronic infusion of angiotensin II (28), and reduced renal mass (18, 19, 21). In regard to 2K1C models of renovascular hypertension, the majority of studies report that a high-NaCl diet does not exaggerate ABP (10, 15, 35, 49, 51). However, these previous studies largely relied on tail-cuff measurements of ABP or ABP measurements at a single time point. A telemetry-based study using a 2K1C rat model reported no significant differences between 0.2% and 4.0% NaCl diets (35). However, the conclusion was based on a single 24-h systolic ABP value at 3 wk despite systolic values appearing greater in rats fed 4.0% versus 0.2% NaCl (188 mmHg vs. 162 mmHg, respectively). Furthermore, the time of dietary salt manipulation may represent another important factor. Previous
studies (10, 15, 35, 49, 51) administered salt diets either at the time of renal artery clipping or after the establishment of hypertension rather than acclimating animals to the respective diet to mimic chronic dietary salt consumption in clinical populations.

The purpose of the present investigation was to evaluate the effects of dietary salt intake on 2K1C hypertension using mice chronically fed 0.1% or 4.0% NaCl diets and monitored with modern ABP telemetric approaches. Animals were fed the respective diets for 2 wk before clipping to produce a steady state and mimic chronic dietary pattern. We hypothesized that elevated ABP due to renal artery stenosis exhibits salt sensitivity, as dietary salt exacerbates several models of hypertension associated with similar characteristics. To assess hemodynamic changes, we assessed in vivo pulse pressure wave velocity (PWV) for aortic stiffness and measured vascular function from conduit and resistance arteries by myography.

MATERIALS AND METHODS

Animals. All experimental procedures conformed to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male C57BL/6J mice (8–15 wk; Jackson Laboratories strain 00664) were housed in a temperature-controlled room (22 ± 1°C), given ad libitum access to deionized water, and randomly assigned to a 0.1% NaCl (Research Diets, D17020) or 4.0% NaCl (Research Diets, D17013) chow diet for 2 wk before experimentation.

2K1C model. 2K1C hypertension was produced with methods described previously (26, 41, 59). Mice were anesthetized with isoflurane (2–3% in 100% oxygen). Through a retroperitoneal incision, the right kidney was gently retracted and a small section of the renal artery isolated free from the renal vein and/or nerve. A 0.5-mm polytetrafluoroethylene (PTFE) catheter (ID: 0.008 × OD: 0.014; Braintree Scientific SUBL140) was cut longitudinally, placed around the renal artery distal to the adrenal artery, and secured with two 10-0 nylon sutures with 2-0 nylon (Braintree Scientific). The catheter tip was advanced 1.5 cm into the femoral artery, and the transmitter body was inserted in the flank. Mice were treated with Buprenex (0.03 mg/kg buprenorphine ip; 2 times per day for 48 h; Henry Schein) and Enroflox (2 mg/kg enrofloxacin ip; Norbrook Laboratories).

Experiment 1: Effect of dietary salt intake on 2K1C hypertension. To assess the impact of a high-salt diet on 2K1C hypertension, male C57BL/6J mice (n = 8 per group) were singly housed and fed a 0.1% or 4.0% NaCl diet for at least 1 wk. Then, mice were anesthetized with isoflurane (2–3% in 100% oxygen) and instrumented with PA-C10 telemetry units (Data Science International) as described previously (41). The catheter tip advanced 1.5 cm into the femoral artery, and the transmitter body was inserted in the flank. Mice were treated with Buprenex (0.03 mg/kg ip; 2 times per day for 48 h) and Enrofloxacin (2 mg/kg ip) and allowed to recover for 1 wk. Then, baseline ABP and heart rate were recorded for 4 days before and 21 days after induction of 2K1C hypertension. The raw ABP signal was sampled and digitized at 500 Hz with a Mikro1401 and Spike2 software (CED). Data were analyzed beat to beat for systolic and diastolic ABP. Beat-to-beat pulse pressure was calculated by subtracting the diastolic pressure from the systolic pressure. Mean ABP was calculated by diastolic plus 1/3 pulse pressure. Daytime BP was measured from 10:00 AM to 4:00 PM (lights on 7:00 AM–7:00 PM), and nighttime BP was measured from 10:00 PM to 4:00 AM (lights off 7:00 PM–7:00 AM). Twenty-four-hour averages were calculated with daytime and nighttime values.

Experiment 2: Effect of dietary salt intake on PWV in 2K1C hypertension. To assess aortic stiffness, beat-to-beat PWV was measured in a second group of 2K1C and sham-operated mice fed a 0.1% or 4.0% NaCl diet. Male C57BL/6J mice (n = 8 per group) were randomly assigned and fed 0.1% and 4.0% NaCl diets. Two weeks later, 2K1C or sham surgery was performed as described above, but telemetry units were not implanted. At 18–20 days after 2K1C or sham surgery, mice were anesthetized with isoflurane (1.8–2.5% in 100% oxygen). Body temperature was maintained at 37.0 ± 0.2°C through a servo-controlled temperature controller and rectal probe (CWE Inc.). A fluid-filled catheter (PE10 tubing; Braintree Scientific) was advanced 0.7 cm into the left common carotid artery, proximal to the bifurcation of the internal/external carotid artery. A second fluid-filled catheter (PE-10) was advanced 1.5 cm into the femoral artery. ABP waveforms were recorded simultaneously with a BPM-832 Dual Pressure Monitor (CWE Inc.). The ECG was recorded through two leads placed subcutaneously on the left and right chest wall. The signal was amplified (100×) and filtered (10–1,000 Hz) with a differential AC amplifier (AM Systems model 1700). Then, mice stabilized at 1.8% isoflurane for 15 min. ABP and ECG waveforms were digitized (1,000 Hz) with a Mikro1401 and Spike2 software. PWV was calculated by the time difference between the upstroke of the carotid versus femoral arterial pressure waves as triggered by the QRS complex of the ECG for 30 consecutive heartbeats. The distance between the carotid and femoral catheter was confirmed postmortem, divided by the carotid-femoral transit time, and reported as meters per second. At the end of experiments, a blood sample (0.2 mL) was collected from the arterial line into a microcentrifuge tube containing heparin (5 U) and centrifuged (10,000 g, 1 min). Plasma electrolytes were assessed with a Medica Electrolyte Analyzer (Na+/K+/Cl−) (Medica Corporation). All measurements were conducted between 10:00 AM and 4:00 PM.

Experiment 3: Assessment of aortic collagen deposition. Male C57BL/6J mice (n = 6 per group) were randomly assigned and fed 0.1% and 4.0% NaCl diets for 2 wk. Then, 2K1C or sham surgery was performed as described above. At 18–21 days after surgery mice were anesthetized with isoflurane (2–3% in 100% oxygen), whole blood was terminally collected by cardiac puncture and centrifuged, and plasma was stored. Aortas were excised and fixed in 4.0% paraformaldehyde (10 mM PBS). Tissue was processed in the Research Histology Core at the University of Pittsburgh. Briefly, aortas were paraffin embedded and serially sectioned (5 μm). Sections were stained with Masson’s trichrome (MTC) and were quantitatively evaluated for collagen deposition. Sections from the thoracic aorta were batch processed and imaged by light microscopy. MTC signal was quantified by color thresholding in Fiji, and MTC-positive area was normalized to the total area of the vascular wall.

Experiment 4: Assessment of vascular function in aorta and mesenteric arteries. Male C57BL/6J mice (n = 8 per group) were randomly assigned and fed 0.1% and 4.0% NaCl diets for 2 wk. Then, 2K1C or sham surgery was performed as described above. At 18–21 days later, mice were anesthetized with isoflurane (2–3% in 100% oxygen). The aortas and mesenteric arteries were rapidly excised, cleaned of fat, and cut into 2-mm rings. Rings were placed on a 2-pin myograph (DMT 620M) filled with physiological salt solution (PSS) containing (in mM) 119 NaCl, 4.7 KCl, 1.17 MgSO4, 1.18 KH2PO4, 5.5 d-glucose, 25 NaHCO3, 0.027 EDTA, and 2.5 CaCl2, pH 7.4 when bubbled with 95% O2-5% CO2 at 37°C. CaCl2 was added once PSS was bubbled. Aorta rings were preconstricted with a cumulative dose response to prostaglandin F2α (1 × 10−7 to 1 × 10−5 M) and mesenteric arteries constricted to doses of prostaglandin mimetic U46619 (1 × 10−7 to 5 × 10−7 M). Vessels were washed three times with PSS and allowed to rest for 30 min. A final wash was performed, and vessels rested for an additional 10 min.

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After the last wash period, aortic rings and mesenteric arteries were constricted with a single dose of prostaglandin F2α (1 × 10^{-9} M) or U46619 (5 × 10^{-7} M), respectively. After plateau was reached, a continuous dose-respons curve of acetylcholine (1 × 10^{-9} to 1 × 10^{-4} M) was used to assess endothelium-dependent relaxation and sodium nitroprusside (1 × 10^{-9} to 1 × 10^{-4} M) to assess endothelium-independent relaxation. Ca^{2+}-free PSS containing 1 × 10^{-6} sodium nitroprusside was added to determine maximal relaxation. Relaxation percentage was normalized by the change in maximal dilation via Ca^{2+}-free PSS. EC_{50} and E_{\text{max}} values were calculated with GraphPad Prism 8 for each animal from a four-parameter nonlinear regression curve. Data were analyzed using software. D’Agostino–Pearson tests were performed for normality. Vasodilatory responses to acetylcholine and sodium nitroprusside were analyzed by comparing EC_{50} or E_{\text{max}} values.

**Statistics.** All data were analyzed with GraphPad Prism v8.0 software. D’Agostino–Pearson tests were performed for normality. Data that passed normality and equal variance tests were analyzed by ANOVA. ABP, PWV, heart rate, renal mass, and plasma electrolytes were compared with a two-way ANOVA (group × time) with repeated measures when appropriate. When significant F values were obtained, post hoc tests were performed with Bonferroni or paired t tests. Vasodilatory responses to acetylcholine and sodium nitroprusside were analyzed by comparing EC_{50} or E_{\text{max}} values. Values were generated for each animal by curve-fit analysis using a four-parameter, nonlinear regression curve. Data were analyzed using a two-way ANOVA (diet × group) with Bonferroni post hoc test for multiple comparisons. Vasoconstrictor responses were analyzed with a two-way ANOVA with Bonferroni post hoc test for multiple comparisons.

Sample sizes were calculated with G*Power (12) and estimated effect sizes from published studies (28, 41). Experiment 1 was designed to detect a ≥10 mmHg change in ABP (α = 0.05, power = 0.9, d = 2; n = 7 or 8 mice/group). Experiment 2 was designed to detect a 1 m/s change in PWV (F test, α = 0.05, power = 0.9, f = 0.7; n = 24 mice, n = 6/group). Experiment 3 was designed to detect a 30% change in collagen deposition (F test, α = 0.05, power = 0.9, f = 0.7; n = 24 mice, n = 6/group). Experiment 4 was designed to detect a 20% change in vessel diameter, which results in a 65% change in vessel flow (F test, α = 0.05, power = 0.9, f = 0.65; n = 28 mice, n = 7/group).

**RESULTS**

Experiment 1: A high-salt diet acutely exaggerates 2K1C hypertension but chronically increases arterial pulse pressure. To assess whether a high-salt diet exacerbates ABP in 2K1C hypertension, mice were fed 0.1% and 4.0% NaCl and ABP was assessed by telemetry. No baseline differences in 24-h mean, systolic, or diastolic ABP were observed between mice fed 0.1% and 4.0% NaCl (Fig. 1). After induction of 2K1C hypertension by unilateral placement of a PTFE catheter around the right renal artery (day 0), ABP significantly increased in mice fed 0.1% and 4.0% NaCl (Fig. 1). A two-way ANOVA of mean ABP revealed a significant effect for salt diet (P = 0.003), time (P = 0.001), and interaction (P = 0.035). Post hoc testing indicated that mean ABP of 2K1C mice fed 0.1% versus 4.0% was significantly higher on days 1–7 of 2K1C hypertension (Fig. 1A). However, there was no difference in mean ABP of 2K1C mice fed 0.1% versus 4.0% NaCl diet at days 9–21. Two-way ANOVA of systolic ABP indicated significant effects for salt diet (P = 0.046), time (P < 0.001), and interaction (P = 0.009) (Fig. 1B). Diastolic ABP exhibited similar significant changes due to 2K1C hypertension for time (P < 0.001) and interaction (P < 0.001) but not salt (P = 0.378) (Fig. 1C). Further analysis indicated that the

**Fig. 1.** High dietary salt acutely exaggerates 2-kidney, 1-clip (2K1C) hypertension but chronically increases arterial pulse pressure. A–D: means ± SD of 24-h mean arterial blood pressure (ABP) (A), systolic ABP (B), diastolic ABP (C), and arterial pulse pressure (D) of mice fed either 0.1% NaCl or 4.0% NaCl diet (n = 8 per group). 2K1C was induced at day 0. A 2-way ANOVA with repeated-measures analysis (time and diet) followed by Bonferroni t tests assessed group differences. *P < 0.05. E: box plot diagram of kidney mass for 2K1C mice fed either 0.1% NaCl or 4.0% NaCl diet (n = 8 per group). Mass of the contralateral kidney was included for comparison. A 2-way ANOVA with repeated-measures analysis (time and diet) followed by Bonferroni t tests assessed group differences. *P < 0.05.
Table 1. Daytime and nighttime hemodynamic data of sham-operated and 2K1C mice fed 0.1% or 4.0% NaCl diet at baseline or at days 7 and 20

|                      | Daytime |                      | Nighttime |
|----------------------|---------|----------------------|-----------|
|                      | Baseline | Day 7 | Day 20 | Baseline | Day 7 | Day 20 |
| Mean ABP, mmHg       | 97 ± 4  | 125 ± 6* | 124 ± 11* | 107 ± 3 | 140 ± 5* | 138 ± 8* |
| ΔMean ABP, mmHg      | 0 ± 3   | 30 ± 8* | 18 ± 11* | 0 ± 5 | 34 ± 7* | 24 ± 11* |
| Systolic ABP, mmHg   | 119 ± 5 | 152 ± 6* | 152 ± 11* | 131 ± 4 | 171 ± 4* | 170 ± 8* |
| Diastolic ABP, mmHg  | 85 ± 4  | 112 ± 7* | 110 ± 10* | 94 ± 3 | 125 ± 6* | 122 ± 9* |
| Heart rate, beats/min | 553 ± 38 | 526 ± 35 | 523 ± 36 | 614 ± 33 | 594 ± 31 | 585 ± 22 |
| Pulse pressure       | 34 ± 1  | 40 ± 1* | 42 ± 1* | 37 ± 1 | 46 ± 2* | 49 ± 1* |
| ΔPulse pressure      | 0 ± 1   | 6 ± 4* | 5 ± 4* | 0 ± 2 | 9 ± 4* | 8 ± 3* |

0.1% NaCl [n = 8 (daytime) and 8 (nighttime)]

|                      |                      |                | 4.0% NaCl [n = 8 (daytime) and 8 (nighttime)] |
| Mean ABP, mmHg       | 93 ± 4              | 136 ± 10** | 121 ± 18* | 109 ± 9 | 150 ± 7** | 137 ± 11* |
| ΔMean ABP, mmHg      | 0 ± 4               | 44 ± 11** | 26 ± 6* | 0 ± 4 | 43 ± 6** | 25 ± 11* |
| Systolic ABP, mmHg   | 115 ± 5             | 165 ± 10** | 154 ± 20* | 131 ± 4 | 182 ± 7** | 174 ± 13* |
| Diastolic ABP, mmHg  | 82 ± 3              | 121 ± 10* | 105 ± 15 | 98 ± 11 | 133 ± 7** | 119 ± 10* |
| Heart rate, beats/min | 512 ± 14            | 517 ± 27    | 507 ± 23 | 598 ± 15 | 588 ± 30 | 562 ± 34 |
| Pulse pressure       | 33.8 ± 0.7           | 44.5 ± 0.7* | 49.4 ± 1.9** | 33.7 ± 3.1 | 49.0 ± 0.7* | 54.8 ± 1.5** |
| ΔPulse pressure      | 0 ± 2               | 11 ± 2* | 13 ± 8** | 0 ± 8 | 15 ± 8* | 14 ± 7** |

Values are means ± SD for 2-kidney, 1-clip (2K1C) and sham-operated mice. ABP, arterial blood pressure. Two-way ANOVA and Bonferroni post hoc test performed for significance. *P < 0.05 from baseline; #P < 0.05 between groups.

Differences in ABP were present during both daytime and nighttime periods (Table 1). Heart rate remained stable throughout the experimental period (Table 1).

2K1C mice fed 0.1% and 4.0% NaCl exhibited a time-dependent increase in pulse pressure (systolic ABP — diastolic ABP) (Fig. 1D). A two-way ANOVA revealed a significant effect of salt diet (P = 0.003), time (P < 0.001), and interaction (P < 0.001). Post hoc testing indicated that the magnitude of the increased pulse pressure was significantly greater in mice fed 4.0% NaCl versus 0.1% NaCl at day 9 and throughout the remainder of the experiment. These differences in arterial pulse pressure were present during both daytime and nighttime periods (Table 1). A two-way ANOVA of kidney mass revealed a significant effect of group (P < 0.001) but not diet (P = 0.591) or interaction (P = 0.415). Post hoc testing indicated that kidney mass of mice fed 0.1% and 4.0% NaCl was significantly lower in clipped versus unclipped kidneys (Fig. 1E). However, there were no differences between mice fed 0.1% and 4.0% NaCl.

Experiment 2: A high-salt diet increases PWV of 2K1C mice. To assess aortic stiffness, we measured beat-to-beat carotid-femoral PWV of 2K1C or sham-operated mice fed either 4.0% or 0.1% NaCl diet (Fig. 2). Three animals were excluded from the analysis because of blood loss during PWV surgery (n = 2) or death after 2K1C surgery (n = 1). A two-way ANOVA revealed a significant effect for group (P = 0.004), salt diet (P = 0.036), and interaction (P = 0.001). Post hoc testing revealed a significant elevation in PWV of 2K1C mice fed 4.0% NaCl versus sham-operated mice fed 4.0% NaCl and 2K1C mice fed 0.1% NaCl (Fig. 2). No significant differences were found between sham-operated mice fed 0.1% versus 4.0% NaCl diet or sham-operated versus 2K1C mice fed 0.1% NaCl.

A two-way ANOVA of mean ABP revealed a significant effect of group (P < 0.0004) but not salt diet (P = 0.742) or interaction (P = 0.980). Mean ABP of 2K1C mice was higher than mean ABP of sham-operated mice; however, there were no differences in mean ABP between 2K1C mice fed 0.1% versus 4.0% NaCl diet. A two-way ANOVA of heart rate revealed no significant effect of group (P = 0.931), salt diet (P = 0.405), and interaction (P = 0.942) (Fig. 2C). A two-way ANOVA of renal mass indicated a significant effect for group (P < 0.001) but not salt diet (P = 0.504) or interaction (P = 0.414). Post hoc testing further revealed significant reductions in renal mass of 2K1C mice fed 0.1% NaCl and 4.0% NaCl versus sham-operated groups (Fig. 2D). Two-way ANOVAs revealed no significant differences in body weight or plasma electrolyte concentrations across groups (Table 2).

Fig. 2. A high-salt diet increases pulse pressure wave velocity (PWV) of 2-kidney, 1-clip (2K1C) mice: box plot diagrams of PWV (A), mean arterial blood pressure (MAP) (B), heart rate [C, beats/min (bpm)], and right kidney mass (D) in sham-operated and 2K1C mice fed either 0.1% NaCl or 4.0% NaCl diet (n = 7 or 8 per group). Data were analyzed by a 2-way ANOVA (condition and diet) followed by Bonferroni post hoc test. *P < 0.05.

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Table 2. Body weight and plasma electrolyte data of sham-operated and 2K1C mice fed 0.1% or 4.0% NaCl diet

|               | Sham, 0.1% | 2K1C, 0.1% | Sham, 4.0% | 2K1C, 4.0% |
|---------------|------------|------------|------------|------------|
| n             | 7          | 8          | 7          | 7          |
| Body weight, g| 24.9 ± 2.7 | 23.5 ± 2.2 | 25.5 ± 3.5 | 23.9 ± 2.1 |
| Na⁺, mM       | 145.7 ± 3.0| 145.8 ± 3.3| 144.5 ± 2.1| 144.6 ± 1.8|
| K⁺, mM        | 4.17 ± 0.49| 3.73 ± 0.32| 4.86 ± 0.76| 4.52 ± 0.30|
| Cl⁻, mM       | 118.0 ± 6.0| 116.6 ± 3.4| 117.8 ± 2.3| 117.8 ± 2.8|

Values are means ± SD for 2-kidney, 1-clip (2K1C) and sham-operated (Sham) mice. A 2-way ANOVA was performed for statistical significance.

**Experiment 3.** Histological assessment of aortic collagen deposition reveals no differences across groups. Aortas collected from a third cohort of mice were histologically assessed for increased collagen deposition typically associated with aortic fibrosis. Figure 3 illustrates representative examples of aortas processed with Masson’s trichrome staining. Quantification of collagen-positive area and two-way ANOVA did not reveal any significant differences for group (P = 0.407), salt diet (P = 0.166), and interaction (P = 0.132).

**Experiment 4:** High-salt diet impairs endothelium-dependent vasodilation in the aorta but not mesenteric arteries of 2K1C mice. Endothelial function is a key modulator of conduit artery and resistance artery function. Thus, we assessed both endothelium-dependent and -independent vasodilation in aortas of sham-operated and 2K1C mice fed 0.1% NaCl or 4.0% NaCl diet. Endothelium-dependent vasodilation was assessed to increasing concentrations of acetylcholine (1 × 10⁻³ to 1 × 10⁻⁴ M). Figure 4, A and B, plot the aorta relaxation responses to acetylcholine and sodium nitroprusside, respectively. The curves were compared by calculating the EC₅₀ and Eₘₐₓ. A two-way ANOVA of acetylcholine EC₅₀ indicated a significant effect for group (P = 0.009), diet (P = 0.006), and interaction (P = 0.010) (Fig. 4C). Post hoc testing revealed a significant increase in the acetylcholine EC₅₀ of 2K1C mice fed 4.0% NaCl versus sham-operated mice fed 0.1% NaCl, sham-operated mice fed 4.0% NaCl, and 2K1C mice fed 0.1% NaCl. Furthermore, a two-way ANOVA of acetylcholine Eₘₐₓ revealed a significant effect for group (P = 0.001) but not diet (P = 0.0744) or interaction (P = 0.308). Post hoc testing indicated that 2K1C mice fed 4.0% NaCl had a significantly lower Eₘₐₓ than 2K1C mice fed 0.1% NaCl or sham-operated mice fed 0.1% or 4.0% NaCl (Fig. 4D). Two-way ANOVAs for sodium nitroprusside EC₅₀ or Eₘₐₓ of aorta revealed no significant differences for diet (P > 0.3), group (P > 0.5), or interaction (P > 0.2) (data not shown).

Figure 5 illustrates vasodilatory responses of third-order mesenteric arteries to acetylcholine and sodium nitroprusside. The curves were compared by calculating the EC₅₀ and Eₘₐₓ. A two-way ANOVA for acetylcholine EC₅₀ indicated no significant differences for diet (P = 0.884), group (P = 0.162), or interaction (P = 0.699) and no significant differences in Eₘₐₓ for diet (P = 0.494), group (P = 0.602), or interaction (P = 0.385) (Fig. 5, C and D). In addition, a two-way ANOVA for sodium nitroprusside EC₅₀ indicated no significant differences for diet (P = 0.159), group (0.769), or interaction (P = 0.892) and no differences in Eₘₐₓ for diet (P = 0.641), group (0.252), or interaction (P = 0.645) (data not shown).

Finally, vasoconstriction responses to KCl administration were assessed in both aorta and mesenteric arteries (Fig. 6). A two-way ANOVA of aortic response revealed a significant main effect for dietary salt (P = 0.040) but not group (P = 0.618) or interaction (P = 0.076). Post hoc testing further revealed a significant elevation in vasoconstriction responses between 4.0% NaCl-fed 2K1C mice and 0.1% NaCl-fed 2K1C control mice (Fig. 6A). A two-way ANOVA of mesenteric responses to KCl revealed no significant differences for dietary salt (P = 0.546), group (P = 0.284), or interaction (P = 0.810) (Fig. 6B).

**DISCUSSION**

The present study tested whether a murine 2K1C model of renovascular hypertension exhibits salt sensitivity and vascular...
dysfunction. Twenty-four-hour telemetry-based recordings of ABP indicate that a high-salt diet transiently exaggerated 2K1C renovascular hypertension. Unexpectedly, arterial pulse pressure increased more in 2K1C mice fed 4.0% versus 0.1% NaCl. PWV measurements confirmed the presence of aortic stiffness in 2K1C mice fed 4.0% but not 0.1% NaCl or sham-operated mice. Interestingly, 2K1C mice fed 4.0% NaCl exhibited a reduced endothelium-dependent vasodilation in the aorta but not in mesenteric resistance arteries. These findings demonstrate that a high-salt diet transiently exaggerates 2K1C renovascular hypertension but chronically promotes aortic stiffness and selective vascular dysfunction.

A high-salt diet contributes to or exaggerates hypertension in many experimental models (19, 21–23, 25, 28, 38). Although previous studies have explored the impact of dietary salt in the 2K1C model, these investigations used tail-cuff measurements, implemented high-salt feeding at the time of or after clipping, and largely relied on single time points for ABP measurements (10, 15, 34, 35, 51). Here, telemetry-based recordings indicate no baseline differences in ABP between mice fed 0.1% and 4.0% NaCl. These observations are consistent with published studies examining the effect of salt diet on ABP in standard laboratory rodent strains regarded as salt resistant (27, 28, 54, 56). Despite the lack of differences in baseline ABP of mice fed 0.1% versus 4.0% NaCl, the mechanisms supporting ABP such as cardiac output or total peripheral resistance could differ between groups. The NaCl content of the diet was chosen to allow direct comparisons to prior studies of salt-sensitive hypertension in rodents (19, 21–23, 25, 28, 38), but we acknowledge that a limitation is whether this level of dietary salt reflects human salt consumption.

Despite the lack of differences in baseline ABP of mice fed 0.1% and 4.0% NaCl, a high-salt diet transiently exaggerated 2K1C hypertension during the first 7 days, and ABP normalized thereafter. Other studies report a similar time course or no differences in ABP between high- and low-NaCl groups (10, 15, 45, 49, 51). The mechanism(s) by which a high-salt diet transiently exaggerates ABP in 2K1C mice is not known but may include a higher cardiac output due to the dietary salt loading, enhanced cardiovascular reactivity by high salt to surgical stress, or an interaction between high salt and RAAS. The development of experimental renovascular hypertension is strongly linked with activation of RAAS (6, 7, 48). This is supported by evidence in which plasma renin activity and angiotensin II levels sharply increase in parallel with ABP immediately after renal artery clipping in 2K1C models (7, 52, 63). RAAS dependence of 2K1C hypertension is further substantiated by significant ABP-lowering effects of renin antibodies or converting enzyme inhibitors administered during the early phase of 2K1C ABP elevation (<2 wk after 2K1C induction) but not the chronic phase that is less sensitive to RAAS inhibition (36, 48). Thus, the exaggerated ABP over the initial 7 days may reflect an interaction between dietary salt and activation of the RAAS.

Surprisingly, a high-salt diet did not produce a sustained salt-sensitive phenotype in 2K1C hypertension. This chronic phase of 2K1C renovascular hypertension is attributed to a small activation of the RAAS and sympathoexcitation (5, 24,
A high-salt diet, by itself, sensitizes sympathetic circuits and exaggerates sympathetic reflex responses (54, 55). Furthermore, multiple models of neurogenic hypertension are salt sensitive (19, 21–23, 25, 28, 38), and we therefore expected the chronic phase of 2K1C hypertension to demonstrate a salt sensitivity. The lack of a sustained salt-sensitive hypertension may reflect a compensatory pressure natriuresis or attenuated RAAS activation in the chronic phase of 2K1C hypertension, but these mechanisms remain to be specifically tested.

An unexpected finding in the present study was the greater increase in arterial pulse pressure and aortic stiffness of 2K1C mice fed 4.0% NaCl versus 0.1% NaCl. This finding was independent of ABP but could be a consequence of other factors (e.g., increased stroke volume). The greatest difference in pulse pressure occurred at day 21 when ABP between groups was similar. Carotid-femoral PWV measurements confirmed the presence of increased aortic stiffness in 2K1C mice fed 4.0% but not 0.1% NaCl. To our knowledge, these findings represent the first report of a novel interaction between renal artery stenosis and dietary salt to promote aortic stiffness. PWV measurements are sensitive to both vascular wall distension and the level of ABP. Surprisingly, 2K1C mice fed 0.1% NaCl did not have an elevated PWV versus sham-operated mice fed 0.1% NaCl. The reason for the lack of a difference is unclear. Nevertheless, our findings are clinically important, as increased aortic stiffness is a predictor of future cardiovascular and total mortality (29, 58) and a determinant of renal microvascular damage (14, 50). Aortic pulse pressure and carotid-femoral PWV positively correlated with increased renal resistance index (measure of altered pulsatile flow-velocity waveform in the renal artery) in hypertensive patients independently of elevated ABP (17, 47). Similarly, a longitudinal study reported that arterial stiffness was significantly elevated in patients with refractory atherosclerotic renovascular hypertension compared with essential hypertensive patients (13). Altogether, the above evidence suggests that dietary salt loading may promote a unique interaction between renovascular hypertension and vascular function.

Both arterial pulse pressure and carotid-femoral PWV measurements indicate that dietary salt and renovascular hypertension promote conduit vessel dysfunction. Using myography, we observed a significant impairment in endothelium-depen-
Dietary salt promotes aortic stiffness in 2K1C hypertension

LI. D., A. C., and S. D. conceived and designed research; L. J. D., S. A. H., and S. D. performed experiments; L. J. D., S. A. H., P. L. K., and S. D. interpreted results of experiments; L. J. D. prepared figures; L. J. D. drafted manuscript; L. J. D., S. A. H., M. M. W., W. B. F., A. C., and S. D. approved final version of manuscript.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L. J. D., A. C., and S. D. conceived and designed research; L. J. D., S. A. H., and S. D. performed experiments; L. J. D., S. A. H., P. L. K., and S. D. interpreted results of experiments; L. J. D. prepared figures; L. J. D. drafted manuscript; L. J. D., S. A. H., P. L. K., M. M. W., W. B. F., A. C., and S. D. approved final version of manuscript.

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A second potential mechanism for the impaired vasodilatory responses is due to bradykinin. Bradykinin influences endothelial nitric oxide synthase activity and renovascular hypertension (2, 33, 53). Pharmacological blockade of the bradykinin-2 receptor in combination with high-salt diet synergistically and negatively influenced conduit artery remodeling (46). Deletion of the bradykinin-2 receptor accelerates and exacerbates 2K1C hypertension, thereby suggesting that bradykinin promotes vascular homeostasis when ABP is elevated (8, 33). Moreover, N(ω)-nitro-L-arginine methyl ester (L-NAME) administration failed to increase ABP in bradykinin-2 receptor-deficient mice with renovascular hypertension (8). Future studies should investigate whether a high-salt diet impacts bradykinin levels or bradykinin-2 receptor expression in 2K1C hypertension. Collectively, our data suggest that the murine 2K1C model exhibits a transient salt-sensitive hypertension during the initial week when peak activation of the RAAS occurs. Unexpectedly, we also found that a high-salt diet plus 2K1C hypertension produced a time-dependent and progressive increase in aortic pulse pressure due to aortic stiffness and impaired endothelial function in conduit vessels. Surprisingly, this effect was specific to the aorta, as mesenteric resistance artery function was preserved. The mechanism supporting the synergistic impact remains to be elucidated. However, this model may represent a unique approach to study hypertensive cardiovascular disease in the absence of peripheral vascular endothelial dysfunction. The findings also highlight potential issues regarding interrogations using aortic rings to reflect changes in vascular function of resistance vessels.

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