High-salt diet blunts renal autoregulation by a reactive oxygen species-dependent mechanism

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Fellner RC, Cook AK, O’Connor PM, Zhang S, Pollock DM, Inscho EW. High-salt diet blunts renal autoregulation by a reactive oxygen species-dependent mechanism. Am J Physiol Renal Physiol 307: F33–F40, 2014. First published May 28, 2014; doi:10.1152/ajprenal.00040.2014.—High dietary salt is common in Western countries and is an important contributor to increased cardiovascular disease. Autoregulation of renal blood flow (RBF) and glomerular filtration rate (GFR) is an essential function of the renal microcirculation that could be affected by excessive dietary salt. High salt (HS) increases renal ROS generation partly by the enzyme NADPH oxidase. We hypothesized that a HS diet would impair autoregulation via NADPH oxidase-dependent ROS generation. The role of NADPH-dependent ROS production on HS diet would impair autoregulation via NADPH oxidase-dependent mechanisms.

An important hemodynamic mechanism of the mammalian kidney is the ability to autoregulate renal blood flow (RBF) and glomerular filtration rate (GFR). Autoregulation of RBF is postulated to involve as many as four mechanisms to stabilize RBF and GFR when challenged with fluctuations in renal arterial pressure (13, 20). The two primary mechanisms of autoregulation are the tubuloglomerular feedback mechanism and the myogenic response, which combine to regulate preglomerular microvascular resistance (7, 13, 19, 20, 26). Autoregulation of RBF plays an important renal protective role by preventing transmission of transient increases of arterial pressure to glomerular capillaries (5, 18). While the existence of the autoregulatory phenomenon is well accepted, the specific mechanisms responsible for transducing changes in renal perfusion pressure (RPP) to changes in preglomerular microvascular resistance remain unresolved.

Lai et al. (14, 16) have shown that increased ROS play a role in HS-induced renal damage in mice with reduced renal mass and that increased ROS generated by NADPH oxidase are likely to be the source for this. ROS reportedly contribute to renal injury in Dahl salt-sensitive hypertensive rats (11, 30). In addition, Dahl salt-sensitive hydrenephrotic rats fed a HS diet show reduced myogenic responses to increased renal arterial pressure, suggesting that ROS could be blunting autoregulation, thereby contributing to renal injury in this model (28). Taken together, these observations led to the hypothesis that a HS diet increases renal ROS accumulation in normal Sprague-Dawley rats and blunts autoregulation of RBF and GFR. Experiments were performed to determine if increased salt intake leads to a ROS-dependent decrease in afferent arteriole autoregulatory behavior using both in vitro and in vivo approaches.

METHODS

Animals. Experiments were performed in 280- to 320-g male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC). All animals were cared for in accordance with National Institutes of Health guidelines, and all procedures were approved by the Institutional Animal Care and Use Committee of Georgia Regents University.

Diet. Rats were divided into four groups. Rats were fed either a normal salt (NS) diet (0.8% NaCl) or a HS diet (8% NaCl) for 14 days and had ad libitum access to tap water. A subset of each group of rats received apocynin in the drinking water at a concentration of 1.5 mM for HS rats (HS + apocynin group) and 4.5 mM for NS rats (NS + apocynin group) such that all rats received ~90 mg·kg–1·day–1 (Table 1). The concentrations in the drinking water differed between HS and NS groups to account for the difference in daily water intake (on average ~105 ml/day for the HS + apocynin group vs. ~37 ml/day for the NS + apocynin group).

EXCESS DIETARY NaCl is linked to hypertension and hypertensive kidney injury, yet many Americans consume more than twice the American Heart Association’s recommended 1.5 g Na+/day (1, 3, 6, 25). A direct correlation exists between reducing Na+ intake and reducing systolic blood pressure (SBP) (23). The mechanisms by which high salt (HS) intake increases blood pressure and/or enhances renal injury in hypertension are poorly understood, but studies have correlated this with increased oxidative stress (29, 34). Several groups have reported that ROS can contribute to the renal injury that occurs with salt-sensitive hypertension (8, 11, 14, 30).

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Table 1. *Body weight, kidney weight, urine flow rate, water consumption, apocynin treatment, and blood pressure data for rats used for in vivo autoregulatory experiments*

<table>
<thead>
<tr>
<th></th>
<th>NS Group</th>
<th>HS Group</th>
<th>NS + Apo Group</th>
<th>HS + Apo Group</th>
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<td>Body weight, g</td>
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<td>312.3 ± 9.8</td>
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<td>Systolic blood pressure, mmHg</td>
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<td>108.5 ± 3.4</td>
<td>107.3 ± 1.9</td>
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<td>106.7 ± 3.6</td>
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<tr>
<td></td>
<td>110.0 ± 1.6</td>
<td>117.8 ± 2.4</td>
<td>105.5 ± 1.8</td>
<td>110.5 ± 1.9</td>
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<tr>
<td>Left kidney weight, g</td>
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<td>1.28 ± 0.05*</td>
<td>1.14 ± 0.06</td>
<td>1.17 ± 0.02*</td>
<td>1.25 ± 0.08*</td>
</tr>
<tr>
<td>Right kidney weight, g</td>
<td>1.03 ± 0.02</td>
<td>1.32 ± 0.05*</td>
<td>1.15 ± 0.05</td>
<td>1.20 ± 0.02*</td>
<td>1.30 ± 0.11*</td>
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<td>Urine flow rate, µl/min</td>
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<td>5.9 ± 0.9</td>
<td>7.5 ± 0.9</td>
<td>12.2 ± 2.5*</td>
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<td>Daily water intake, ml</td>
<td>Ad libitum</td>
<td>Ad libitum</td>
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<td>105.1 ± 4.6*</td>
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<td>Apocynin daily intake, mg·kg body wt⁻¹·day⁻¹</td>
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<td>N/A</td>
<td>88.2 ± 1.4</td>
<td>89.6 ± 3.9</td>
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</tbody>
</table>

Value are means ± SE. Rats were divided into the following groups. Rats were fed either a normal salt (NS) diet (0.8% NaCl) or a high-salt (HS) diet (8% NaCl) for 14 days and had ad libitum access to tap water. A subset of each group of rats received apocynin in the drinking water at a concentration of 1.5 mM for HS rats (HS + Apo group) and 4.5 mM for NS rats (NS + Apo group). N/A, not applicable. *P < 0.05, significant vs. the NS group.

ml/day for the NS + apocynin group; Table 1). Apocynin was dissolved in ethanol (0.17 g/ml) and added to the drinking water accordingly, yielding a final ethanol concentration of 0.15% for HS rats and 0.45% for NS rats. A subset of rats on HS was given vehicle (0.15% ethanol), and there was no effect on autoregulatory function.

**Measurement of blood pressure.** SBP was measured in conscious rats by tail-cuff plethysmography (IITC, Woodland Hills, CA) on days 0, 7, and 13 of dietary salt supplementation. Recorded SBP was averaged from four recordings on each measurement day.

In *vitro* blood-perfused juxtamedullary nephron preparation. Videomicroscopy experiments were performed in vitro using the blood-perfused juxtamedullary nephron technique, as previously described (9). Apocynin (100 µM) was superfused across the inner cortical surface of the prepared kidney. To assess autoregulatory function, afferent arteriolar diameters were measured during a control period at a RPP of 100 mmHg and then at pressures ranging from 65 to 170 mmHg stepped in 15-mmHg increments over successive 5-min periods. Afferent arteriolar diameter was calculated from the average of measurements taken at a single site at 12-s intervals during the last 2 min of the 5-min pressure step.

In *vivo* autoregulation of GFR and RBF. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip, Abbott Laboratories, North Chicago, IL) and placed on a servo-controlled heating table to maintain body temperature (37–38°C) as measured by a rectal probe (Physitemp, Clifton, NJ). A tracheotomy tube [polyethylene (PE-205)] was inserted into the trachea to ensure a patent airway. The left femoral artery and right carotid artery were cannulated (PE-50) for continuous recording of arterial pressure both below (femoral) and above (carotid) an adjustable aortic clamp. A catheter was inserted into the jugular vein to maintain plasma volume by infusion of 6% BSA (10 µl/min, Sigma, St. Louis, MO) in PBS and to infuse FITC-inulin (30 mg/ml, 10 µl/min, Sigma) in PBS.

The abdomen was opened by a midline incision. A segment of the aorta between the left and right renal arteries was separated from the surrounding connective tissue and vena cava. An adjustable vascular occluder was placed around the aorta to control left renal artery pressure as monitored by the femoral artery cannula. The left renal artery was separated from the renal vein, and an ultrasonic flow probe (MA1PRB, Transonic Systems, Ithaca, NY) was installed to measure RBF. Mean arterial pressure (carotid and femoral) and RBF were continuously recorded by an eight-channel Powerlab (AD Instruments, Colorado Springs, CO). The ureters were individually cannulated (PE-10), and urine was collected. Urine volume was determined gravimetrically. Upon completion of the surgical procedures, rats were allowed to stabilize for 60 min before the experimental protocol began (22). GFR was calculated as the clearance of FITC-labeled inulin. The FITC-inulin concentration was determined fluorometrically (FLx800, BioTek, Winooski, VT).

**Analysis of renal tissue peroxidation.** Formalin-fixed kidneys were embedded in paraffin, sectioned (5.0-µm slices), and prepared for the assessment of lipid peroxidation as indicated by increased levels of 4-hydroxy-2-monenal (4-HNE) as an index of regional oxidative stress (2, 21, 31, 35, 36). Briefly, paraffin-embedded kidney sections were warmed for 30 min (60°C) and then deparaffinized in xylene for 15 min. After sections had been rehydrated through a series of increasingly dilute ethanol solutions (100–70% ethanol then distilled water), they were washed in distilled water (10 min) and then steamed in antigen retrieval solution (IHC-Tek epitope retrieval solution, 40 min, 60°C, IHCWORLD, Ellicott City, MD). After sections were blocked with 10% rabbit serum solution for 30 min, 4-HNE staining was detected by treating the tissue slices overnight (4°C) with anti-4-HNE primary mouse monoclonal antibody (1:600 dilution, Japan Institute for the Control of Aging, Shizouka, Japan). After incubation with primary antibody, samples were incubated (30 min) with biotinylated rabbit anti-mouse secondary antibody (1:400, Dako, Carpinetria, CA) and development with avidin-biotinylated horseradish peroxidase complex (30 min, Vectastain ABC-AP Kit, Vector Lab, Burlingame, CA), each prepared according to the manufacturer’s instructions. Finally, sections were stained using a BCIP/NBT kit for 20 min (Alkaline Phosphatase Substrate Kit IV, Vector Labs) following the manufacturer’s instructions. All dilutions and washing steps were performed with PBS (pH 7.4). Prepared sections were evaluated by light microscopic image analysis (>5 magnification) using ImageJ software to quantify relative staining intensity across all treatment groups.

**Western blot analysis of p67 phox expression.** Rat kidneys were flushed with Tyrode buffer containing 5.2% BSA, sectioned longitudinally, and snap frozen in liquid nitrogen before the renal cortex was homogenized in RIPA buffer in the presence of PMSF (1 mM, Cell Signaling Technology, Danvers, MA). The protein concentration in the lysates was determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein for each sample were denatured in sample buffer and separated by electrophoresis on Bolt 4–12% bis-Tris gels (Invitrogen, Carlsbad, CA). Equal amounts of protein for each sample were denatured in sample buffer and separated by electrophoresis on Bolt 4–12% bis-Tris gels (Invitrogen, Carlsbad, CA). Separated protein bands were transferred to nitrocellulose membranes and blocked in 5% nonfat dry milk in Tris-buffered saline + 0.1% Tween 20 (pH 7.4, 60 min) at room temperature, and membranes were then incubated with mouse anti-p67phox primary antibody (1:2,000, Millipore, Temecula, CA) at 4°C overnight. Blots were washed (3 times, 10 min) in Tris-buffered saline + 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:4,000, 60 min) at room temperature. After being washed with Tris-buffered saline + 0.1% Tween 20, membranes were visualized by enhanced chemiluminescence (Clarity Western ECL Substrate, Bio-Rad), and images were developed after exposure to X-ray film (CLASSIC X-Ray Film, Research Products, Prospect, IL). The same
blots were stripped, washed, and reprobed with monoclonal anti-β-actin (1:10,000, Sigma) and again imaged using chemiluminescence.

**Experimental protocol.** The protocol used consisted of six 20-min collection periods where urine was collected from the right and left kidneys to determine urine volume and flow rate. A femoral artery blood sample was taken at the midpoint of each period for the measurement of FITC-inulin concentration. The first two 20-min collection periods were control periods to determine baseline mean arterial pressure, urine flow rate, RBF, and GFR. At the start of the third period, the aortic occluder was tightened until femoral artery pressure declined to 95 mmHg, where it was maintained throughout the next 60 min (third to fifth periods). At the beginning of the sixth period, the vascular occluder was opened to restore full blood flow and perfusion pressure below the clamp. A RPP of 95 mmHg was chosen because it is near the lower limit of the renal autoregulatory range reported for anesthetized rats (22). The autoregulatory index (AI) was calculated according to following formula: AI for RBF = [(RBF1 - RBF2)/RBF1]/[(RPP1 - RPP2)/RPP1], where RBF1 and RPP1 are the averages for those values during the two control periods and RBF2 and RPP2 are the averages of the last two (steady state) periods of reduced RPP. An AI of zero indicates perfect autoregulation, and an AI of 1 indicates the absence of autoregulation.

**Statistical analyses.** Data are presented as means ± SE for *n* = 7–12 rats/group. RBF and GFR data were analyzed by two-way repeated-measures ANOVA followed by (Bonferroni’s) post hoc test to compare between groups. 4-HNE and p67phox data were analyzed by one-way ANOVA followed by a Newman-Keuls post hoc test to compare between groups. *P* values of ≤0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

**RESULTS**

**HS blunts the autoregulatory behavior of afferent arterioles in vitro.** The afferent arteriole responses to apocynin treatment and to changes in perfusion pressure in NS and HS rats are shown in Figs. 1 and 2, respectively. Under control conditions with RPP set at 100 mmHg, afferent arteriole diameters from NS rats and 14-day HS rats were similar and averaged 15.8 ± 0.2 and 14.5 ± 1.0 µm, respectively (Fig. 1). Reducing perfusion pressure to 65 mmHg resulted in similar increases in afferent arteriolar diameter for both groups, as indicated by a 12 ± 1% (17.6 ± 0.2 µm, *P* < 0.05) increase in diameters of afferent arterioles from NS rats and a 12 ± 2% increase in afferent arterioles from HS rats (16.2 ± 1.0 µm, *P* < 0.05; Fig. 2, *A* and *B*). Pressure-mediated autoregulatory vasoconstriction was significantly attenuated in arterioles from HS rats compared with arterioles from NS rats. Diameters of arterioles from HS rats at 170 mmHg (15.3 ± 1.4 µm) were statistically similar to their baseline diameters, whereas diameters of arterioles from NS rats were significantly reduced to 74 ± 2% of

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**Fig. 1.** Baseline afferent arteriolar diameter in kidneys at a renal perfusion pressure (RPP) of 100 mmHg using the in vitro juxtamedullary nephron technique before and during topical administration of apocynin (Apo) in the superfusate. Rats were divided into the following groups. Rats were fed either a normal salt (NS) diet (0.8% NaCl) or a high-salt (HS) diet (8% NaCl) for 14 days and had ad libitum access to tap water. A subset of each group of rats received Apo in the drinking water at a concentration of 1.5 mM for HS rats (HS + Apo group) and 4.5 mM for NS rats (NS + Apo group). Data are expressed as means ± SE; *n*, number of afferent arterioles studied.

**Fig. 2.** Afferent arteriolar diameter response to changing RPP in kidneys using the in vitro juxtamedullary nephron technique. *A*: responses of NS, HS, NS + Apo, and HS + Apo groups. *B*: data expressed as a percentage of the control diameter. The autoregulatory profile ranged from 65 to 170 mmHg. Data are expressed as means ± SE; *n*, number of afferent arterioles studied. *P* < 0.05, HS vs. NS groups; +*P* < 0.05, HS vs. HS + Apo groups.
Apoacynin restores autoregulatory behavior of afferent arterioles from HS kidneys. Acute apoacynin treatment had no significant effect on baseline afferent arteriole diameter in kidneys from rats on a HS diet (15.0 ± 0.4 μm before vs. 15.3 ± 0.6 μm after) and was similar to those from rats on NS (15.8 ± 0.2 μm) or HS (14.5 ± 1.0 μm) diet (Fig. 1). Treatment of arterioles with apoacynin normalized autoregulatory behavior in afferent arterioles from HS rats such that arteriolar diameter declined to 12.6 ± 0.7 μm or 82 ± 3% of control when RPP was increased to 170 mmHg (Fig. 2, A and B). Apoacynin treatment did not alter the autoregulatory response of arterioles from NS rats (Fig. 2, A and B). These data indicate that acute apoacynin treatment normalizes afferent arteriole autoregulatory behavior in rats fed HS.

**HS diet significantly increases RBF but not GFR or SBP.** Additional experiments were conducted to determine whether high dietary salt impairs autoregulation at the whole kidney level. Figures 3A and 4A show the effects of a 14-day HS diet on RBF and GFR, respectively. At ambient RPP (first and second periods), RBF was significantly higher in HS rats (12.01 ± 0.5 vs. 8.6 ± 0.9 ml/min for NS rats, P < 0.05; Fig. 3A), whereas GFR was similar (Fig. 4A) between the two dietary groups. Additionally, both urine flow rate and kidney weight in HS rats were significantly increased compared with NS rats (P < 0.05; Table 1). SBP was similar for all groups on day 1 of NS or HS diet and did not change significantly during the 14 days of salt, apoacynin, or vehicle treatment (Table 1).

**HS diet blunts renal autoregulation of RBF and GFR in vivo.** Figure 3, A and B, shows the effect of reducing RPP on RBF. When the aortic clamp was tightened to reduce RPP to 95 mmHg, RBF significantly declined in HS rats by 21 ± 1% from a baseline of 12.0 ± 0.5 to 9.5 ± 0.5 ml/min compared with no change in NS rats (from 8.6 ± 0.9 to 8.7 ± 1.0 ml/min, P < 0.05; Fig. 3A). The AI for NS rats was −0.01 ± 0.32, indicating efficient autoregulatory behavior. The AI calculated for HS rats averaged 1.38 ± 0.22, indicating a significant loss of autoregulation (P < 0.05; Fig. 3C).

Figure 4, A and B, shows the effect of a HS diet on autoregulation of GFR. As shown in Fig. 4A, HS rats exhibited a significant decrease in GFR when RPP was reduced from ambient to 95 mmHg. Normalized GFR was significantly reduced by 28 ± 2% from baseline in HS rats (462 ± 24 to 333 ± 26 μl·min⁻¹·g body wt⁻¹, P < 0.05), whereas NS rats exhibited only a 3 ± 0% (507.0 ± 47.0 to 490 ± 41 μl·min⁻¹·g body wt⁻¹) reduction in GFR. These results show that a HS diet blunts autoregulation of both RBF and GFR in vivo.

**Chronic apoacynin restores blunted autoregulation of RBF in HS rats.** Treatment of HS rats with apoacynin had no effect on renal excretory function or SBP (Table 1). Figure 3, A and B, shows the effects of apoacynin in the drinking water on RBF.
Autoregulation. Baseline RBF was similar in the HS + apocynin rats compared with HS rats (12.5 ± 0.5 vs. 12.1 ± 0.5 ml/min; Fig. 2A). However, as shown in Fig. 3, A and B, reducing RPP to 95 mmHg revealed significantly improved RBF autoregulation in HS + apocynin rats compared with HS rats alone. HS + apocynin rats exhibited only a 9 ± 0% decrease in RBF from baseline (12.5 ± 0.5 to 11.3 ± 0.5 ml/min) compared with the 21 ± 1% reduction in RBF observed in HS rats (P < 0.05; Fig. 3B). Assessment of the AI for these groups (Fig. 3C) revealed that autoregulation was improved by ~50% from 1.4 ± 0.2 for HS rats to 0.7 ± 0.2 for HS + apocynin rats (P < 0.05). Vehicle treatment of HS rats had no effect on RBF autoregulation, with AI determined to be 1.37 ± 0.25. These data suggest that ROS generation by NADPH oxidase contributes to the blunted RBF autoregulatory response observed in rats fed a HS diet.

Apocynin restores blunted autoregulation of GFR in HS rats. As shown in Fig. 4, A and B, apocynin also significantly improved autoregulation of GFR. GFR declined by just 10 ± 1% in HS + apocynin rats compared with 28 ± 2% in HS rats (Fig. 4B). Apocynin treatment had no significant effect on autoregulation of GFR in NS rats (473 ± 37 µl·min⁻¹·g body wt⁻¹ at 110 mmHg compared with 419 ± 22 µl·min⁻¹·g body wt⁻¹ at 95 mmHg; Fig. 4A). From these findings, we conclude that apocynin-sensitive ROS production is involved in HS-blunted autoregulation of GFR.

HS diet increases renal lipid peroxidation and p67phox expression. 4-HNE was measured in the renal cortex and medulla to determine the extent of lipid peroxidation in kidneys from rats on NS and HS diets with and without apocynin treatment. As shown in Fig. 5A, HS increased renal cortical

**Fig. 4.** Glomerular filtration rate (GFR) responses to decreasing RPP in kidneys of NS and HS rats with and without Apo in the drinking water (~90 mg·kg⁻¹·day⁻¹). A: GFR normalized to body weight. The autoregulatory profile ranged from ambient to 95 mmHg left kidney RPP. BW, body weight. B: data normalized to starting GFR over the autoregulatory profile range from ambient to 95 mmHg left kidney RPP. Data are expressed as means ± SE; n, number of rats studied. *P < 0.05, HS vs. NS groups; +P < 0.05, HS vs. HS + Apo groups.

**Fig. 5.** Effect of HS diet and chronic oral Apo treatment on 4-hydroxy-2-nonenal (4-HNE) levels in the renal cortex and medulla. 4-HNE staining intensity is presented as arbitrary units across groups. *P < 0.05 vs. kidneys from the NS group; +P < 0.05 vs. kidneys from the HS + Apo group; #P < 0.05 vs. kidneys from the NS + Apo group.
lipid peroxidation by nearly 40% compared with kidneys from NS rats (P < 0.05). Apocynin treatment suppressed the increase in renal cortical 4-HNE staining (P < 0.05) in the HS + apocynin group to levels similar to the NS and NS + apocynin groups while having no significant effect in kidneys from NS rats.

Similar analysis of the renal medulla indicated that lipid peroxidation was significantly higher in the medulla than in the cortex (P < 0.05) but was not significantly influenced by 14 days of HS diet. Chronic apocynin treatment reduced lipid peroxidation in the renal medulla similarly in both NS and HS groups (Fig. 5B). These data support the contention that HS diet increases oxidative stress in the rat renal cortex and that chronic apocynin treatment effectively suppresses that effect.

Western blot analysis of rat cortical tissue lysates revealed that p67phox expression was significantly increased in kidneys from rats fed a HS diet (P < 0.05; Fig. 6) compared with kidneys from NS rats. Apocynin treatment prevented the increase in p67phox expression (P < 0.05) in the HS + apocynin group to levels similar to the NS and NS + apocynin groups while having no significant effect in kidneys from NS rats. These data suggest that the HS diet increases NOX2 expression in the renal cortex.

**DISCUSSION**

The present study demonstrates that a HS diet blunts renal autoregulation in vivo and in vitro and increases renal lipid peroxidation and p67phox expression. Furthermore, apocynin, a NADPH oxidase inhibitor, restores normal renal autoregulation in kidneys from HS rats both in vivo and in vitro while at the same time suppressing the increase in renal lipid peroxidation and p67phox expression. Accordingly, these data indicate that excessive dietary salt consumption can lead to blunted renal autoregulation derived in part through ROS generated by a NOX2-dependent mechanism.

There is strong evidence that the Western diet, which includes excessive salt consumption, is a major risk factor for hypertension and renal microvascular dysfunction in a subset of humans (1, 6, 23), and similar effects have been observed in animal models (1, 6, 23). In the present study, afferent arterioles from HS rats exhibited blunted pressure-mediated adjustments in vessel diameter, consistent with markedly reduced autoregulatory reactivity. In the in vivo setting, autoregulatory behavior was also impaired rendering kidneys unable to maintain a stable RBF or GFR, which is commonly observed in normal rat kidneys when perfusion pressure is reduced within the autoregulatory range. Importantly, autoregulatory behavior was normalized in kidneys from HS rats either acutely (in vitro) exposed to the NADPH oxidase inhibitor apocynin or chronically treated (in vivo) with apocynin. These data suggest that high dietary salt stimulates ROS generation by NADPH oxidase activation, which leads to blunted renal autoregulatory capability. These findings are consistent with our general hypothesis that impairment of autoregulatory efficiency could be an important contributor to the renal injury associated with long-term dietary salt excess and salt-sensitive hypertension. The data also suggest that prevention of renal oxidative stress or blunting of ROS signaling pathways represent potentially important therapeutic targets for reducing or preventing renal injury.

Lai et al. (15) examined the impact of high dietary salt on myogenic autoregulatory reactivity using isolated, perfused murine afferent arterioles. They reported that HS caused reduced generation of vascular wall tension and an impaired ability to constrict in response to increased luminal perfusion pressure (15). In addition, high dietary salt blunted the myogenic autoregulatory behavior of afferent arterioles of hydronephrotic kidneys of normotensive Dahl salt-resistant rats, which are devoid of functional tubules (28). The experiments presented here extend those observations by demonstrating that autoregulatory behavior is blunted after 2 wk of high dietary salt when tested in a model with a full complement of functional tubules such that the nephrovascular unit is maintained. As shown in Fig. 2, a HS diet impairs the afferent arteriole response to increases in perfusion pressure, indicated by maintenance of an essentially static diameter over a pressure range from 110 to 170 mmHg. Seemingly, there was some increase in smooth muscle tension generation in response to increased RPP as the arterioles did not passively increase in diameter; however, the arterioles did fail to decrease in diameter over that pressure range like control arterioles from NS rats.

The in vivo experiments presented here provide important confirmation of the in vitro experiments and demonstrate that high dietary salt blunts whole kidney autoregulation of both GFR and RBF. Additionally, chronic HS intake will necessitate increased salt excretion to maintain salt balance and to prevent the development of hypertension. Increased salt excretion could be achieved by increased glomerular filtration and/or decreased tubular solute and water reabsorption. We observed that both HS and HS + apocynin rats had larger kidneys (Table 1) and had baseline RBF ~50% higher than NS rats (Fig. 3A), consistent with previously published results (4, 24, 32).
contrast, GFR was not increased in HS rats compared with NS rats (Fig. 4A), which is consistent with other published results (24), suggesting that filtration fraction was lower for HS rats. Nevertheless, renal oxidative stress was certainly elevated given the increase in lipid peroxidation and increase in p67phox expression. Indeed, Feng et al. (8) reported that p67phox expression, but not that of the other subunits tested, increased in the renal medulla of Dahl salt-sensitive rats fed a HS diet for 7 days. It is notable that apocynin treatment improved autoregulatory function in vivo and in vitro and that it prevented or suppressed the increased lipid peroxidation and p67phox expression, respectively, consistent with reduction of oxidative stress and its functional consequences.

While it is clear that high dietary salt leads to blunted autoregulatory behavior, the mechanisms that underlie this effect are unclear. Lai et al. (16, 17) reported that increasing perfusion pressure in isolated afferent arterioles from normal mouse kidneys resulted in a p47phox-dependent increase in superoxide production, which enhanced myogenic responses, whereas H$_2$O$_2$ impaired pressure-induced contractions independent of the myogenic mechanism. These data are in contrast with our observation that apocynin had no effect on autoregulatory responses of NS rat kidneys in vitro or in vivo. The reason for this discrepancy remains unclear but could reflect differences in species or experimental preparations. In other work from the same group, myogenic reactivity was significantly attenuated in afferent arterioles from mice with reduced renal mass and fed either NS or HS diets for 3 mo (15).

Additionally, a study (37) in Dahl salt-sensitive rats showed that ROS are upregulated in response to a HS diet in conjunction with hypertension and that blockade of NADPH oxidase with N-acetyl-L-cysteine leads to a reduction in blood pressure. Therefore, the present study focused on determining the role of NADPH oxidase-derived ROS in the blunted renal autoregulatory behavior observed in a salt-resistant strain of rats fed a HS diet. The results of the present study extend those previous findings by strongly implicating NADPH-dependent ROS generation in attenuating autoregulatory efficiency under HS conditions. Inhibition of NADPH oxidase in vitro restored the HS blunted ability of the afferent arterioles to respond normally to increases in RPP.

Use of apocynin as an inhibitor of NADPH oxidase has come into question given observations that it can also act as a free radical scavenger in cultured cells at concentrations as low as 100 μM, whereas others have seen none of these effects at concentrations as high as 300 μM (10, 27). Some of the variability in these observations could reflect the in vitro cell systems used and their inability to oxidize apocynin into its active form, diapocynin (12). We performed our in vitro experiments using an apocynin concentration of 100 μM, placing us at the lower limit of where free radical scavenging capabilities for apocynin have been reported. Estimations of our in vivo plasma concentration of apocynin from oral administration of 90 mg·kg$^{-1}$·day$^{-1}$ are more difficult to predict because the apocynin was administered to rats in the drinking water and their water intake was distributed across the 24-h drinking cycle. Wang et al. (33) reported that a 50 mg/kg intragastric bolus of apocynin in a rat produced an peak plasma apocynin concentration of no more than 7.23 μM 7 min after ingestion followed by a decline to ~1.5 μM 20 min after the bolus. The half-life for apocynin in the plasma was ~6 min, and the bioavailability of apocynin across the gut was ~2.8%.

Accordingly, we applied their findings to estimate the highest apocynin concentration that might be expected in the extracellular fluid of our apocynin-treated rats. Using the 2.8% bioavailability Wang et al. reported for bolus intragastric apocynin administration, we estimate an extracellular fluid apocynin concentration of ~76 μM if the entire daily apocynin intake was given as an oral bolus and was immediately absorbed by the gut. Understandably, our rats consumed the apocynin throughout the day, representing considerably slower apocynin delivery, which would most likely result in apocynin concentrations well below levels found to have radical scavenging effects. Irrespective of the specificity of apocynin, it is clear that ROS, whatever the source, contribute to the impaired autoregulation in rats fed a HS diet.

We reproduced the in vitro observations to the in vivo setting to determine the impact of HS-induced ROS production on kidney function and whole kidney autoregulation. Apocynin treatment had no detectable effect on salt-associated increases in kidney weight and RBF but substantially improved autoregulation of RBF and GFR in HS + apocynin rats. These HS-induced effects of ROS on autoregulatory function could participate in both positive and negative roles in modulating GFR and RBF responses to changes in pressure.

In summary, this study provides compelling new evidence for the hypothesis that high dietary salt can negatively impact rat renal autoregulatory capability. This impaired function appears to involve ROS derived from NADPH oxidase because inhibition of NADPH oxidase with apocynin normalized or improved autoregulatory behavior in vitro and in vivo. These observations also underscore the potential importance of regulating salt intake. It is interesting to speculate that in a setting of high dietary salt, the increase in RBF and blunted autoregulatory behavior could represent a physiological response to facilitate increased salt excretion to restore, or maintain, salt balance.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

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