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Carolina Panico, Zaiming Luo, Sara Damiano, Francesca Artigiano, Pritmohinder Gill and William J. Welch

*Hypertension* 2009, 54:1291-1297: originally published online October 5, 2009  
doi: 10.1161/HYPERTENSIONAHA.109.134783

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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# Renal Proximal Tubular Reabsorption Is Reduced In Adult Spontaneously Hypertensive Rats

## Roles of Superoxide and Na<sup>+</sup>/H<sup>+</sup> Exchanger 3

Carolina Panico, Zaiming Luo, Sara Damiano, Francesca Artigiano, Pritmohinder Gill, William J. Welch

**Abstract**—Proximal tubule reabsorption is regulated by systemic and intrinsic mechanisms, including locally produced autocoids. Superoxide, produced by NADPH oxidase enhances NaCl transport in the loop of Henle and the collecting duct, but its role in the proximal tubule is unclear. We measured proximal tubule fluid reabsorption ( $J_v$ ) in WKY rats and compared that with  $J_v$  in the spontaneously hypertensive rat (SHR), a model of enhanced renal superoxide generation. Rats were treated with the NADPH oxidase inhibitor apocynin (Apo) or with small interfering RNA for p22<sup>phox</sup>, which is the critical subunit of NADPH oxidase.  $J_v$  was lower in SHR compared with Wistar-Kyoto rats (WKY; WKY:  $2.3 \pm 0.3$  vs SHR:  $1.1 \pm 0.2$  nL/min per millimeter;  $n=9$  to  $11$ ;  $P < 0.001$ ). Apo and small interfering RNA to p22<sup>phox</sup> normalized  $J_v$  in SHRs but had no effect in WKY rats.  $J_v$  was reduced in proximal tubules perfused with S-1611, a highly selective inhibitor of the Na<sup>+</sup>/H<sup>+</sup> exchanger 3, the major Na<sup>+</sup> uptake pathway in the proximal tubule, in WKY rats but not in SHRs. Pretreatment with Apo restored an effect of S-1611 to reduce  $J_v$  in the SHRs (SHR+Apo:  $2.9 \pm 0.4$  vs SHR+Apo+S-1611:  $1.0 \pm 0.3$  nL/min per millimeter;  $P < 0.001$ ). However, because expression of the Na<sup>+</sup>/H<sup>+</sup> exchanger 3 was similar between SHR and WKY rats, this suggests that superoxide affects Na<sup>+</sup>/H<sup>+</sup> exchanger 3 activity. Direct microperfusion of Tempol or Apo into the proximal tubule also restored  $J_v$  in SHRs. In conclusion, superoxide generated by NADPH oxidase inhibits proximal tubule fluid reabsorption in SHRs. This finding implies that proximal tubule fluid reabsorption is regulated by redox balance, which may have profound effects on ion and fluid homeostasis in the hypertensive kidney. (*Hypertension*. 2009;54:1291-1297.)

**Key Words:** proximal reabsorption ■ superoxide ■ Tempol ■ apocynin ■ hypertension

In the kidney, the proximal tubule (PT) reabsorbs 60% to 70% of filtered NaCl and fluid. Therefore, changes in PT reabsorption can have profound effects on renal and body fluid balance and may contribute to the development of hypertension. The normal kidney protects against acute increases in blood pressure by excreting NaCl rapidly. The PT is thought to mediate much of this pressure-natriuresis response. In young spontaneously hypertensive rats (SHRs), before the onset of hypertension, expression of the major Na<sup>+</sup> transport systems in the PTs was higher<sup>1</sup> and Na<sup>+</sup> excretion was lower compared with normotensive rats (Wistar-Kyoto [WKY]).<sup>2</sup> This was accompanied by an increase in fluid reabsorption in the PT in young (5-week-old) prehypertensive SHRs compared with WKY. These observations suggest that an exaggerated NaCl and fluid reabsorption in the PT may contribute to the development of hypertension in young SHRs, which persists in the adult animal. However, the increased reabsorption seen in young animals is not consistently observed in adult SHRs. For example, in 7- and 12-week-old SHRs, at a time when hypertension was estab-

lished, baseline proximal tubule fluid reabsorption ( $J_v$ ) in the PT was lower compared with that in WKY rats.<sup>3</sup>  $J_v$  was also lower in adult stroke-prone SHRs<sup>4</sup> compared with WKY rats. This may be related to increased reactive oxygen species, because direct microperfusion of reducing agents corrected this dysfunction.<sup>4</sup> The SHR is indeed an established model of oxidative stress, with elevated superoxide levels and NADPH oxidase (NOX) expression in both vascular and renal tissue.<sup>5</sup>

The uncertain role of the PT is partially attributed to the difficult technologies in measuring function. PT transport in SHRs has been investigated primarily in vitro with cell preparations or in vivo by free-flow micropuncture. However, free-flow measurements, which have characterized PT function on both WKY rats and SHRs, are complicated by the interruption of flow to the macula densa, which tends to overestimate PT flow. Therefore, direct measurement of  $J_v$  by in situ microperfusion is the most accurate method to characterize PT function, because this method separates transport from the confounding effects of flow and tubuloglomerular feedback. Therefore, we measured  $J_v$  in the PTs of hyperten-

Received April 16, 2009; first decision May 9, 2009; revision accepted September 10, 2009.

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*Hypertension* is available at <http://hyper.ahajournals.org>

DOI: 10.1161/HYPERTENSIONAHA.109.134783

sive and normotensive rats by direct microperfusion and recollection to test the hypothesis that increased production of superoxide ( $O_2^{\cdot-}$ ) impairs PT function during hypertension in the adult SHR.

## Materials and Methods

### Animal Preparation

Groups of SHRs and aged-matched WKY rats were pretreated with apocynin (Apo; 16 mg/kg per day), small interfering RNA (siRNA; 25  $\mu$ g, IV) or vehicle (IV) 2 days before experimentation. The Georgetown Animal Care and Use Committee approved the use of rats in this study. On day 3, animals were anesthetized with thiobarbital (Inactin, 80 g/kg IP; Research Biochemicals, Inc) and prepared for *in vivo* micropuncture studies. Cannulae were placed in a jugular vein for infusion of fluids and in a femoral artery for the recording of mean arterial pressure (MAP; Powerlab, AD Instruments Inc). A tracheotomy tube was inserted. The animals were allowed to breathe room air spontaneously. A catheter was inserted in the bladder and another in the left ureter to collect urine. The left kidney was exposed by a flank incision and stabilized in a Lucite cup (Vestavia) mounted on a heated surgical table and bathed in mineral oil maintained at 37°C. After surgical preparation, rats were infused with a solution of 0.154 mol/L of NaCl and 1% albumin at 1.5 mL/h to maintain euvoolemia.<sup>6</sup> Studies were begun after 60 minutes of stabilization.

### Microperfusion of PTs

The PT site was identified, as described previously,<sup>7</sup> by injections from a "finding" pipette containing dye-stained artificial tubular fluid. The flow was blocked by injection of T grease (T grade, Apiezon Products) via a micropipette (10 to 12  $\mu$ m OD) proximal to the perfusion site. The tubule was perfused with a micropipette (8 to 10  $\mu$ m OD) connected to a microperfusion pump (model A1400, World Precision Instruments Inc) at  $18 \pm 3$  nL/min. The perfusion solution contained  $^{14}C$  inulin (New England Nuclear) as volume marker and 0.1% FD&C green dye for identification of the perfused loops. Tubules were perfused for 2 to 8 minutes before fluid collections, which were made at a downstream site with a micropipette (7 to 10  $\mu$ m OD) after placement of a column of oil to block downstream flow. Samples were collected for 3 to 5 minutes and transferred into a constant-bore capillary tube for which the length was measured with a micrometer to calculate the tubular fluid volume.<sup>8</sup> Thereafter, the samples were injected into scintillation fluid and the  $^{14}C$  activity counted. Collected samples with <95% and >105% of microperfused inulin were discarded. The amount of microperfused inulin was estimated by the average of  $^{14}C$ -activity in 4 samples microperfused directly into capillary bore tubes at the end of the experiment. To determine the lengths of the perfused segments, tubules were filled with high-viscosity microfil (Flow Tech, Inc). At the end of the experiments, the kidney was partially digested in 20% NaOH, and the casts were measured under a dissecting microscope. The  $J_v$  was calculated by the difference in the perfusion rate and the collection rate factored by the length of the nephron:  $J_v = V_{\text{perf}} (\text{nanoliters per minute}) - V_{\text{coll}} (\text{nanoliters per minute}) / \text{PT length (millimeters)}$  where  $V_{\text{perf}}$  indicates perfusion rate and  $V_{\text{coll}}$  indicates collection rate and expressed as nanoliters per minute per millimeter. The composition of the perfusion fluid was as follows in mM: 125 NaCl, 20  $\text{NaHCO}_3$ , 5 KCl, 1  $\text{MgSO}_4$ , 2 CaCl, 1  $\text{NaHPO}_4$ , 5 glucose, and 4 urea. The *in vivo* nephron microperfusion technique allows for the control of flow rate,  $\text{Na}^+$  concentration, osmolality, and extracellular pH. This method is limited by the lack of endogenous agents generated in the PT, because all of the perfusions are with artificial tubular fluid containing various antagonists, but it more directly measures the intrinsic transport properties of the epithelium and allows for the use of various inhibitors to test the roles of key players in the regulation of PT function.

In separate nephrons, proximal and distal tubule flows were measured by free-flow micropuncture collections. In these nephrons, a collecting pipette was inserted into a midproximal or distal tubule,

and a small volume of mineral oil was inserted. The oil was allowed to move downstream, and tubular fluid was aspirated into the collection pipette for 3 to 4 minutes. The fluid was transferred to a capillary bore tube and measured by microscopic calibration.

### Small Interference RNA

The siRNA to rat p22<sup>phox</sup> (NM-024160) was validated *in vitro*, as reported previously.<sup>9</sup> The target site in p22<sup>phox</sup> cDNA of the constructs selected is 299 to 320 (AAATTACTACGTCCGGGCTGT). The nonsilencing control siRNA sequence ATTCTCCGAACGTGTACCGT (catalogue No. 1022076; Qiagen) has no homology to any sequence in the mammalian genome. TransIT In-Vivo gene delivery system (Mirus) was used to complex the p22<sup>phox</sup> siRNA to polymer as per the manufacturer's recommendations. This complexed siRNA was brought to 6-mL volume and delivered via the jugular vein in 7 seconds. Kidney cortex was harvested after 48 hours and saved for gene analysis. In a separate group of SHRs, PTs were microdissected for the measurement of mRNA.

### Microdissection of PTs

The kidneys were perfused through the abdominal aorta with 10 mL of cold dissection solution to rinse away the blood. The composition of the dissection solution was as follows, in mM: 135.0 NaCl, 50 KCl, 1.0  $\text{NaH}_2\text{PO}_4$ , 1.2  $\text{MgSO}_4$ , 2.0  $\text{CaCl}_2$ , 6.0 L-alanine, 10.0 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and 5.5 glucose, as well as 0.1% BSA. The kidneys were then perfused with 10 mL of cold dissection solution containing 0.1% collagenase (CLS II, Worthington). Thin sagittal sections were cut from the perfused kidneys and incubated in dissection solution containing 0.1% collagenase at 37°C for 30 minutes during which the solution was bubbled with 100% oxygen. Microdissection of individual PT segments was performed in cold dissection solution under a stereomicroscope.<sup>10</sup>

### RNA Extraction, cDNA Synthesis, and Real-Time PCR

The kidney cortex and PTs were harvested after 48 hours, and RNA was extracted using an RNAqueous-4PCR kit (Ambion). The cDNA was synthesized using superscript III with a random hexamer (Invitrogen). The gene expression for p22<sup>phox</sup> was assessed with real-time PCR (ABI 7700), using a FAM (6-carboxy-fluorescein dye)-labeled p22<sup>phox</sup> Taqman probe assay (Rn00577357\_m1, ABI) multiplexed with a VIC (fluorescein dye)-labeled 18S control probe. Relative amounts of mRNA, normalized by 18S rRNA, were calculated from threshold cycle numbers ( $C_T$ , ie,  $2^{-\Delta\Delta C_T}$ ).

### SDS-PAGE Gel Electrophoresis and Western Analysis

The kidney cortex was placed in radioimmunoprecipitation assay lysis buffer containing the following protease inhibitors: 100  $\mu$ g/mL of PMSF, 5  $\mu$ g/mL of leupeptin, 5  $\mu$ g/mL of aprotinin, and 1 mmol/L of sodium fluoride per milliliter. The dissected sections with radioimmunoprecipitation assay lysis buffer were homogenized in FastPrep Bio101 (Thermo), and the tubes were spun at 12 500 rpm for 15 minutes in a cold centrifuge. The supernatant was aliquoted and frozen at  $-80^\circ\text{C}$  for future analysis. Protein concentrations were determined by Bio-Rad Protein Assay Reagent (Bio-Rad). Protein lysate (100  $\mu$ g) for each sample was denatured in boiling water for 5 minutes. After denaturation, the lysate was placed on ice for 5 minutes and loaded onto a 12.5% SDS-PAGE gel (Bio-Rad). The gel was transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% Blotto milk. The primary antibody for 2  $\mu$ g/mL (ab22765) of  $\text{Na}^+/\text{H}^+$  exchanger 3 (NHE3) was from Abcam Inc, and the antibody for NHE3 exchanger regulatory factor (NHERF) 2 was the kind gift of Drs James B. Wade and Paul A. Welling (University of Maryland, Baltimore, MD). The secondary antibodies (1:10 000) were peroxidase-labeled goat antirabbit (KPL). The blots were probed with  $\beta$ -actin (Sigma) for equal loading.

**Table. MAP and Renal Parameters**

Variable	MAP, mm Hg	GFR, mL/min	VPT, nL/min	VDT, nL/min	UV, $\mu$ L/min
WKY (n=6 to 10)	100 $\pm$ 5	1.9 $\pm$ 0.3	13.9 $\pm$ 0.9	6.5 $\pm$ 1.2	4.6 $\pm$ 0.2
SHR (n=6 to 9)	185 $\pm$ 6*	1.7 $\pm$ 0.5	12.2 $\pm$ 0.9	5.9 $\pm$ 0.8	4.4 $\pm$ 0.5
WKY+Apo (n=4 to 7)	95 $\pm$ 3	2.3 $\pm$ 0.6	13.3 $\pm$ 0.5	5.5 $\pm$ 0.8	4.0 $\pm$ 0.3
SHR+Apo (n=4 to 7)	190 $\pm$ 6*	2.0 $\pm$ 0.5	8.3 $\pm$ 0.5*	5.3 $\pm$ 0.3	4.5 $\pm$ 0.5

GFR indicates glomerular filtration rate;  $V_{PT}$ , proximal tubule flow;  $V_D$ , distal tubule flow;  $U_v$ , urine flow. Each parameter was analyzed by 2 $\times$ 2 ANOVA.

\* $P$ <0.001.

Densitometry for Western analysis was performed with the Image J program (National Institutes of Health) for NHE3 and NHERF2.

### Statistical Analysis

The effects of Apo, siRNA to p22<sup>phox</sup>, Tempol, and S-1611 in WKY rats and SHRs were analyzed by 2 $\times$ 2 ANOVA, with post hoc testing. Values are mean $\pm$ SE.  $P$ <0.05 was considered statistically significant.

### Protocols

#### Protocol 1

First,  $J_v$  in the PT was measured in WKY rats and age-matched SHRs treated with vehicle or Apo (an NOX inhibitor; 16 mg/kg per day) for 48 hours (groups 1 to 4: WKY+vehicle; WKY+Apo; SHR+vehicle; SHR+Apo).

Second,  $J_v$  in the PT was measured in an additional SHR group that had a clamp placed around the aorta proximal to the left renal artery to maintain renal perfusion pressure at 122 $\pm$ 6 mm Hg (SHR+clamp; n=4).

Third, kidneys were harvested to measure protein expression of NHE3 and NHERF2 in the renal cortex.

Fourth, the acute effects of the O<sub>2</sub><sup>-</sup> dismutase mimetic Tempol and the antioxidant Apo microperfused into the PT were tested in WKY rats and SHRs.

#### Protocol 2

The effect of the inhibition of NHE3 was tested by direct microperfusion of S-1661 (10<sup>-5</sup> M), a highly selective inhibitor to NHE3 in groups 1 to 4 above.

#### Protocol 3

$J_v$  of the PT was measured in WKY rats and SHRs treated with siRNA directed to p22<sup>phox</sup>, a critical subunit of NOX or scrambled siRNA (scr). Groups 5 to 8 are as follows: WKY+scr; WKY+siRNA; SHR+scr; SHR+siRNA. Solutions (6 mL) containing siRNA were injected into cannulated jugular veins of anesthetized (1% isoflurane) rats. Animals were allowed to recover and prepared for microperfusion analysis after 48 hours.

## Results

MAP measured under anesthesia was higher in SHRs compared with WKY rats (Table). Treatment for 2 days with Apo had no effect on MAP in either strain. Proximal flow, measured by free-flow collections in separate nephrons, was similar between WKY rats and SHRs but was decreased by Apo in SHRs only ( $P$ <0.001). Distal flow and urine flow were similar in all of the groups.

#### Protocol 1

First, absolute fluid reabsorption ( $J_v$ ) measured in the PT averaged 2.3 $\pm$ 0.3 nL/min in normotensive WKY rats (Figure 1). However,  $J_v$  was lower in PTs of SHRs (1.1 $\pm$ 0.2 nL/min per millimeter;  $P$ <0.001). Apo, an inhibitor of NOX, had no

effect on  $J_v$  in WKY rats but restored  $J_v$  to normal levels in SHRs.

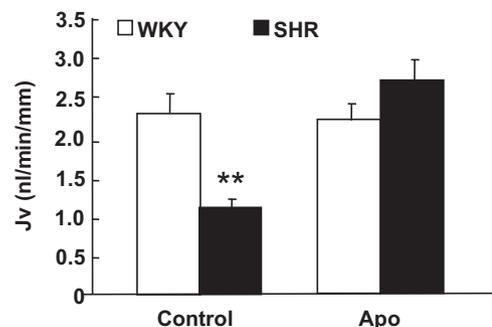
Second, in a separate SHR group, renal perfusion pressure was maintained at 122 $\pm$ 6 mm Hg by an aortic clamp (SHR+clamp).  $J_v$  was unaffected by the acute reduction in renal perfusion pressure (SHR: 1.1 $\pm$ 0.2 vs SHR+clamp: 1.3 $\pm$ 0.3 nL/min per millimeter; n=6 to 7;  $P$  value not significant). This suggests that the differences in  $J_v$  between the 2 strains were not directly related to MAP.

Third, Figure 2 shows that the protein expression of NHE3 in the renal cortex was similar in SHRs compared with WKY rats. NHE3 in the renal cortex is exclusively expressed in the PT. NHERF2 is a postsynaptic density protein-95-disc large-zonula occludens-1 domain-binding element that is associated with reduced NHE-3 activity and translocation.<sup>11</sup> NHERF2 expression in the renal cortex was higher in SHRs compared with WKY rats (Figure 2), suggesting that reduced NHE3 activity in SHRs is attributed to overexpression of this regulatory factor. Indeed, NHERF-2 expression in SHRs was reduced by Apo, further implicating this pathway in NHE3 dysregulation in SHRs.

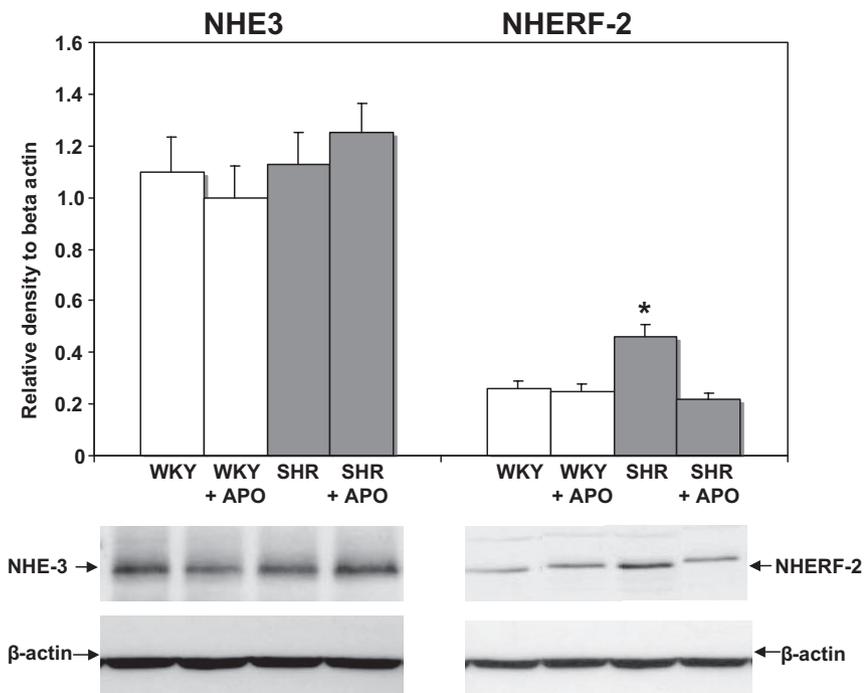
Fourth Figure 3 shows that direct microperfusion of Tempol (10<sup>-4</sup> M) had no effect in PTs of WKY rats but normalized  $J_v$  in PTs of SHR (WKY: 2.3 $\pm$ 0.5 vs WKY+Tempol: 2.4 $\pm$ 0.5 nL/min per millimeter, n=6 to 9 tubules,  $P$  value not significant; SHR: 1.2 $\pm$ 0.3 vs SHR+Tempol: 2.4 $\pm$ 0.5 nL/min per millimeter, n=7 to 10 tubules,  $P$ <0.001). Similarly, acute microperfusion of Apo (10<sup>-5</sup> M) also restored  $J_v$  in SHRs (SHR: 1.2 $\pm$ 0.3 vs SHR+Apo: 2.0 $\pm$ 0.3 nL/min per millimeter;  $P$ <0.05; n=8).

#### Protocol 2

To determine the mechanism of this dysfunction, we tested the activity of NHE3, a major Na<sup>+</sup> uptake path in the PT.  $J_v$



**Figure 1.** Absolute fluid absorption ( $J_v$ ) in PT of WKY rats (n=9 to 11) and SHRs (n=9 to 11); effects of systemic Apo (16 mg/kg per day for 2 days). \*\* $P$ <0.001.

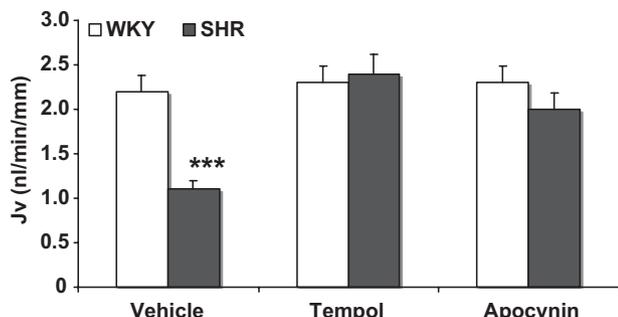


**Figure 2.** Effect of Apo (2 days) on NHE3 and NHERF2 expression in the renal cortex of WKY rats and SHRs. \* $P < 0.01$ .

was reduced by direct microperfusion of the NHE3 selective inhibitor, S-1611 ( $10^{-5}$  M, maximally effective dose) in WKY rats as expected, but not in SHRs (Figure 4). This suggests that the dysfunction is attributable to the reduction in NHE3 activity. When the reduced  $J_v$  of SHRs was restored by Apo, direct microperfusion of S-1611 lowered  $J_v$ , similar to its effect in WKY rats (Figure 5). This suggests that the dysfunction of NHE3 is linked to NOX-generated  $O_2^{\cdot-}$ .

**Protocol 3**

To confirm that the reduction in  $J_v$  of the PT was linked to NOX, we repeated these experiments in rats treated with siRNA direct to p22<sup>phox</sup>, the critical subunit of NOX. mRNA expression of p22<sup>phox</sup>, relative to expression of 18S, was reduced by siRNA treatment by  $62 \pm 4\%$  ( $n=6$ ) in the renal cortical tissue and by  $66 \pm 7\%$  in microdissected PTs ( $n=4$ ; Figure 6).  $J_v$  in SHRs was restored by siRNA to p22<sup>phox</sup> but unaffected in WKY rats (Figure 7). MAP was not affected by siRNA to p22<sup>phox</sup> (MAP scr siRNA:  $118 \pm 3$  mm Hg vs MAP siRNA p22<sup>phox</sup>:  $115 \pm 3$  mm Hg;  $P$  value not significant).

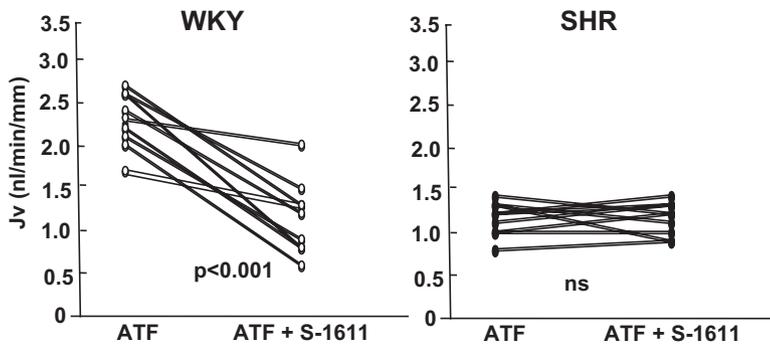


**Figure 3.** Effect of microperfusion of Tempol ( $10^{-4}$  M) or Apo ( $10^{-5}$  M) directly into PT on  $J_v$  in SHRs and WKY rats ( $n=7$  to 9). \*\*\* $P < 0.001$ .

**Discussion**

The major new finding of this study is that fluid reabsorption in the PT of adult SHRs is impaired compared with that in WKY rats. In addition, this reduced function can be completely restored by inhibition of  $O_2^{\cdot-}$ . Treatments that targeted  $O_2^{\cdot-}$  in general (Tempol) and the more specific source of  $O_2^{\cdot-}$ , NOX (Apo, siRNA), restored the lower  $J_v$  of the PT in SHRs. Two results suggest that these effects are independent of the high blood pressure in SHRs. First, Apo and siRNA to p22<sup>phox</sup> did not lower BP in SHRs, yet  $J_v$  was corrected. Second, clamping of the renal artery in SHRs to reduce renal perfusion pressure to normotensive levels had no effect on the reduced  $J_v$ .

To evaluate the mechanism of the reduced  $J_v$ , PTs were perfused with S-1611, a highly selective inhibitor of NHE3. S-1611 reduced  $J_v$  in WKY PT by 50% but, surprisingly, had no effect on the reduced  $J_v$  in SHRs. However, when  $J_v$  was restored in SHR nephrons with Apo, subsequent microperfusion of S-1611 reduced  $J_v$  by 50%, similar to its effect in WKY rats. Because there was no difference in the expression of NHE3 in the renal cortex between strains, these data suggest that the difference in  $J_v$  between SHRs and WKY rats is linked to NHE3 activity rather than content. NHE3 activity may depend on the NHERFs. We showed that NHERF2 expression was higher in SHRs than WKY rats, which may account for reduced NHE3 activity in SHRs. In addition, Apo reduced NHERF2 expression, consistent with restoration of PT function. However, both Apo and Tempol microperfused acutely into the PT restored  $J_v$  in the SHR. This suggests that  $O_2^{\cdot-}$  also regulates  $J_v$  directly and perhaps independently of NHE3 and NHERF2. Combined, these results suggest that  $J_v$  is regulated by a complex interaction of regulatory factors (NHERF2), which translocates NHE3, and more acutely by  $O_2^{\cdot-}$  inactivation of NHE3. The acute effect of  $O_2^{\cdot-}$  may not



**Figure 4.** Jv in PT of WKY rats (n=8) and SHRs (n=7): effects of PT microperfusion of the NHE3 inhibitor, S-1611.

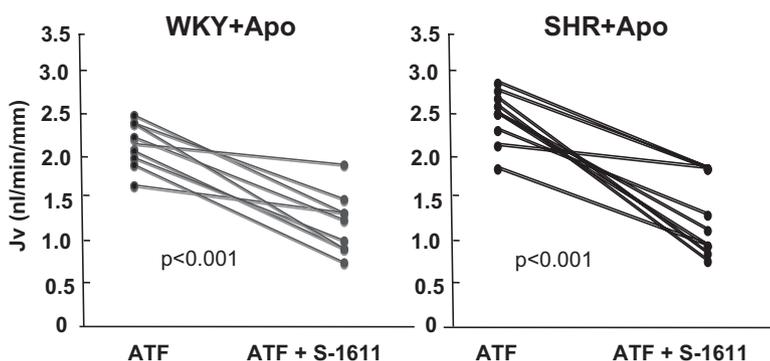
be because of translocation of NHE3, but further studies will be required to determine this mechanism.

Young SHRs have an enhanced proximal tubular reabsorption and sodium uptake,<sup>1</sup> based on reports of greater NHE3 activity and lower  $\text{Na}^+$  excretion. Also,  $J_v$  measured by split-drop micropuncture, which measures the time of injected fluid uptake in the PT, was also higher in 5-week-old SHRs.<sup>3</sup> These studies have popularized the concept that  $\text{Na}^+$  retention in young SHRs contributes to the development of hypertension. However, this overactive PT does not persist into adulthood. The reduced  $J_v$  in the PT of SHRs in this study contrasts with the findings in other studies that did not detect differences in PT flow or reabsorption between adult SHRs and WKY rats.<sup>12</sup> In fact, many studies do not report differences in  $\text{Na}^+$  excretion or transporter activities between adult SHRs and normotensive rats. However, direct measurements have shown that  $J_v$  is lower in adult hypertensive rats compared with normotensive rats.<sup>4</sup> Therefore, our observations with microperfusion and recollection in the SHR confirm these earlier studies that used the split-drop methodology. In addition, Wu and Johns<sup>4</sup> showed that the reduced  $J_v$  could be restored by local delivery of exogenous  $\text{O}_2^{\cdot-}$  dismutase. We confirmed this observation using the membrane-permeant antioxidant Tempol and Apo. In addition, we have extended the studies on the role of  $\text{O}_2^{\cdot-}$  in regulation of PT function by NHE3 and NHERF2 expression.

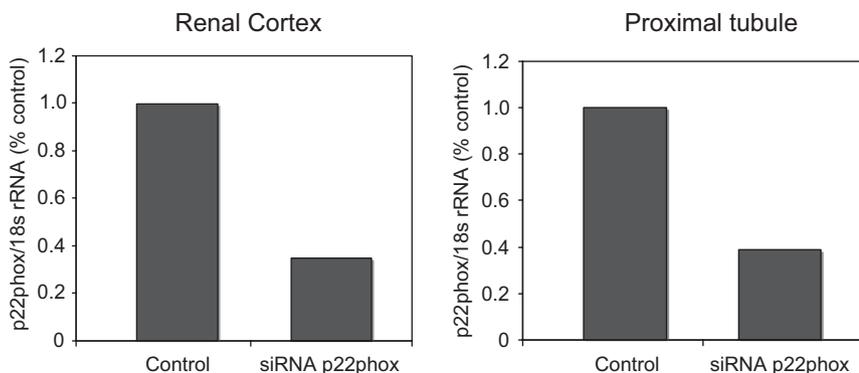
As noted above, PT flow was similar in SHRs compared with normotensive rats.<sup>12</sup> Indeed, we also failed to see differences in PT flow between WKY rats and SHRs when measured by free-flow collections in separate nephrons (Table). However, when  $\text{O}_2^{\cdot-}$  was suppressed by Apo, PT flow was substantially reduced in the SHR PT, consistent with increased  $J_v$  in this group. The failure to detect basal

differences in PT flow in hypertension remains unclear. This could be because of the method used in this study, which blocks flow to the macula densa and tends to increase glomerular filtration rate and, therefore, PT flows in both strains. In addition, similar flow in this segment could be real, and the differences in transport function between these strains occur in segments not evaluated in this study.

How is the PT regulated by  $\text{O}_2^{\cdot-}$ ? One possibility is the interaction of  $\text{O}_2^{\cdot-}$  and NO. NO inhibits  $\text{Na}^+$  uptake in the thick ascending loop of Henle<sup>13</sup> via its effects on cGMP and on the density of membrane channels. However, NO promotes  $\text{Na}^+$  and/or fluid reabsorption in the PT.<sup>14</sup>  $J_v$ , measured by the same method in this study, was lower in inducible NO synthase (NOS) and neuronal NOS knockout mice.<sup>15,16</sup>  $J_v$  in normal rat PT is also reduced by NOS inhibition.<sup>17</sup> Taken together, these results suggest that endogenous NO enhances fluid transport in the PT.<sup>18</sup> Therefore,  $\text{O}_2^{\cdot-}$ , which reduces bioavailable NO, should inhibit fluid reabsorption in the PT, which is consistent with our finding. However, NOS expression in the PT is not consistently shown; therefore, the source of NO is not known. However, there is considerable evidence that PT is constantly exposed to NO, because NOS is expressed in adjacent tissue and cells. In the kidney, endothelial NOS is expressed predominantly in the renal vascular endothelial cells.<sup>19,20</sup> Also, neuronal NOS is expressed in the epithelium, Bowman's capsule, and especially in the macula densa,<sup>21-24</sup> which is in close contact with the PT in the renal cortex. Inducible NOS is widely expressed in the tubule epithelia, including the PT, the thick ascending limb of Henle's loop, and the distal convolute tubule.<sup>19,24</sup> Data supporting the effects of NO to enhance  $\text{Na}^+$  uptake are difficult to obtain, because systemic administration of NOS inhibitors increases blood pressure and renal perfusion pres-



**Figure 5.** Jv in WKY rats (n=9) and SHRs (n=7) after Apo: effects of microperfusion of the NHE3 inhibitor, S-1611.



**Figure 6.** p22<sup>phox</sup> mRNA expression in renal cortex and in microdissected proximal tubules: effects of systemic injection of siRNA directed to p22<sup>phox</sup> 48 hours before tissue collection (n=3 to 6).

sure.<sup>25–27</sup> However, an acute dose of an inducible NOS inhibitor, but not a neuronal NOS inhibitor, both of which did not increase MAP, increased Na<sup>+</sup> excretion 2-fold in rats.<sup>28</sup> Also, we showed that microperfusion of the PT with a nonselective NOS inhibitor reduced J<sub>v</sub> by 30% to 40%, suggesting that NO promotes Na<sup>+</sup> uptake in the PT.<sup>17</sup>

Another possibility suggested by our data is that O<sub>2</sub><sup>•-</sup> inhibits NHE3 activity. NHE3 is a major Na<sup>+</sup> uptake mechanism in the PT, and increased activity of NHE3 in the renal brush border membrane may be involved in the pathogenesis of hypertension.<sup>29–32</sup> However, in elegant experiments, Yip et al<sup>33</sup> and Sneddon et al<sup>34</sup> showed that NHE3 is inactivated by translocation. Therefore, reduced reabsorption in PT of SHR may be attributed to a relocation of the NHE3, which would alter function.<sup>34,35</sup> We showed that, in the SHR, NHE3 activity is suppressed and can be restored by several strategies. Therefore, it is possible that high renal levels of O<sub>2</sub><sup>•-</sup> lead to translocation of NHE3, and antioxidants reactivate this pathway. In addition, the regulatory factor for NHE3, NHERF2, may participate in the redox regulation of NHE3. Increased NHERF2 leads to translocation and inactivation of NHE3.<sup>36,37</sup> We confirmed that NHERF2 is higher in the SHR.<sup>38</sup> This increase is because of higher levels of O<sub>2</sub><sup>•-</sup>, because Apo reduced NHERF2 levels and increased NHE3 expression in the renal cortex and improved J<sub>v</sub> in the PT in the SHR.

Renal levels of O<sub>2</sub><sup>•-</sup> and NOX are higher in SHR compared with WKY rats.<sup>39,40</sup> In addition, in this study we show that p22<sup>phox</sup> mRNA is expressed in microdissected PTs and is reduced by systemic infusion of siRNA to p22<sup>phox</sup>. Also, direct microperfusion of Tempol and Apo into the PT

suggests that the increased O<sub>2</sub><sup>•-</sup> in the SHR is derived from NOX in the PT. Consistent with this view, we showed that the SHR kidneys also had greater expression of NOS, yet bioavailable NO was lower in SHR.<sup>41</sup> Treatment with Tempol increased NO function and presumably NO levels, indicating the powerful scavenging effects of O<sub>2</sub><sup>•-</sup>.

Additional explanation of these results is required, because Na<sup>+</sup> and fluid excretion of age-matched SHR and WKY rats are similar.<sup>12</sup> We suggest that downstream sites of Na<sup>+</sup> reabsorption are increased by O<sub>2</sub><sup>•-</sup>, which compensates for the dysfunction in the PT. Ortiz and Garvin<sup>13</sup> have shown that O<sub>2</sub><sup>•-</sup> increased Na<sup>+</sup> reabsorption in the thick ascending loop of Henle and the collecting duct, which could normalize excretion. The shift in nephron relative function can potentially alter oxygen consumption, because the PT is more O<sub>2</sub> efficient for Na<sup>+</sup> transport than downstream segments. Therefore, the increased O<sub>2</sub><sup>•-</sup> in SHR may contribute to the reduced oxygen efficiency and renal hypoxia seen in this strain.<sup>40</sup>

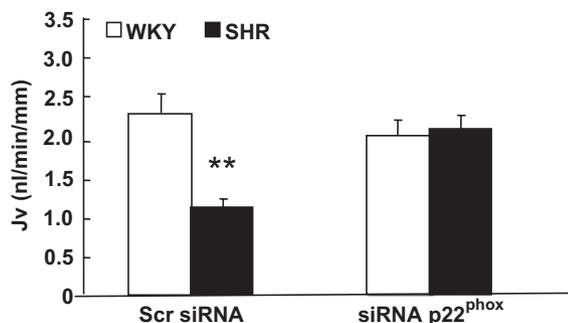
In conclusion, fluid uptake in the PT of adult SHR is impaired because of increased NOX-dependent O<sub>2</sub><sup>•-</sup>. Our data suggest that O<sub>2</sub><sup>•-</sup> reduces NHE3 activity, possibly because of increased expression of the NHE3 regulatory factor, but also inhibits fluid uptake independent of these pathways. These data suggest that increased reactive oxygen species alters proximal tubular reabsorption during hypertension and impacts renal regulation of fluid and solute balance.

## Perspectives

These data suggest that, during hypertension, responsibility for Na<sup>+</sup> and fluid reabsorption along the nephron changes. Reabsorption in the PT is reduced and is increased downstream. This has an impact on oxygen usage for Na<sup>+</sup> uptake, because the PT is relatively more efficient than more distal segments. Therefore, to preserve normal Na<sup>+</sup> excretion, the kidney burns more oxygen as it transfers work during a chronic increase in blood pressure. This may be a normal adaptation as the kidney acts to maintain stable Na<sup>+</sup> balance. Increased O<sub>2</sub><sup>•-</sup> generated during hypertension mediates this shift and, therefore, may be an adaptive process that allows the kidney to preserve normal function at the expense of more oxygen usage.

## Sources of Funding

The studies reported in this article were supported by the National Institutes of Health grants HL089583 and DK072183.



**Figure 7.** J<sub>v</sub> in PT of WKY rats (n=6) and SHR (n=9): effects of siRNA to p22<sup>phox</sup> (systemic injection 48 hours before experimentation). \*\*P<0.001.

## Disclosures

None.

## References

- Aldred KL, Harris PJ, Eitle E. Increased proximal tubule NHE-3 and H<sup>+</sup>-ATPase activities in spontaneously hypertensive rats. *J Hypertens*. 2000;18:623–628.
- Boer PA, Morelli JM, Figueiredo JF, Gontijo JA. Early altered renal sodium handling determined by lithium clearance in spontaneously hypertensive rats (SHR): role of renal nerves. *Life Sci*. 2005;76:1805–1815.
- Thomas D, Harris PJ, Morgan TO. Age-related changes in angiotensin II-stimulated proximal tubule fluid reabsorption in the spontaneously hypertensive rat. *J Hypertens*. 1988;6(suppl):S449–S451.
- Wu XC, Johns EJ. Interactions between nitric oxide and superoxide on the neural regulation of proximal fluid reabsorption in hypertensive rats. *Exp Physiol*. 2004;89:255–261.
- Chabrashvili T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT, Fujita T, Welch WJ, Wilcox CS. Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. *Hypertension*. 2002;39:269–274.
- Welch WJ, Wilcox CS. Potentiation of tubuloglomerular feedback in the rat by thromboxane mimetic: role of macula densa. *J Clin Invest*. 1992;89:1857–1865.
- Kulick A, Panico C, Gill PS, Welch WJ. Low salt intake increases adenosine type 1 receptor expression and function in the rat proximal tubule. *Am J Physiol Renal Physiol*. 2008;295:F37–F41.
- Wilcox CS, Welch WJ, Schreiner GF, Belardinelli L. Natriuretic and diuretic actions of a highly selective adenosine A1 receptor antagonist. *J Am Soc Nephrol*. 1999;10:714–720.
- Modlinger P, Chabrashvili T, Gill PS, Mendonca M, Harrison DG, Griendling KK, Li M, Raggio J, Wellstein A, Chen Y, Welch WJ, Wilcox CS. RNA silencing in vivo reveals role of p22phox in rat angiotensin slow pressor response. *Hypertension*. 2006;47:238–244.
- Tojo A, Tisher CC, Madsen KM. Angiotensin II regulates H<sup>+</sup>-ATPase activity in rat cortical collecting duct. *Am J Physiol*. 1994;267:F1045–F1051.
- Wade JB, Welling PA, Donowitz M, Shenolikar S, Weinman EJ. Differential renal distribution of NHERF isoforms and their colocalization with NHE3, ezrin, and ROMK. *Am J Physiol Cell Physiol*. 2001;280:C192–C198.
- Arendshorst WJ, Beierwaltes WH. Renal tubular reabsorption in spontaneously hypertensive rats. *Am J Physiol*. 1979;237:F38–F47.
- Ortiz PA, Garvin JL. Superoxide stimulates NaCl absorption by the thick ascending limb. *Am J Physiol Renal Physiol*. 2002;48:F957–F962.
- Wang T. Nitric oxide regulates HCO<sub>3</sub><sup>-</sup> and Na<sup>+</sup> transport by cGMP-mediated mechanism in the kidney proximal tube. *Am J Physiol*. 1997;272:F242–F248.
- Wang T, Inglis FM, Kalb RG. Defective fluid and HCO<sub>3</sub><sup>-</sup> absorption in proximal tubule of neuronal nitric oxide synthase-knockout mice. *Am J Physiol Renal Physiol*. 2000;279:F518–F524.
- Wang T. Role of iNOS and eNOS in modulating proximal tubule transport and acid-base balance. *Am J Physiol Renal Physiol*. 2002;283:F658–F662.
- Nie J, Lu Q, Chen S, Wilcox CS, Welch WJ. Regulation of fluid reabsorption in the proximal tubule: role for nitric oxide, ADMA and DDAH [abstract]. *J Am Soc Nephrol*. 2008;19:23A.
- Wang T, Inglis FM, Kalb RG. Defective fluid and HCO<sub>3</sub><sup>-</sup> absorption in proximal tubule of neuronal nitric oxide synthase-knockout mice. *Am J Physiol Renal Physiol*. 2000;279:F518–F524.
- Tojo A, Welch WJ, Bremer V, Kimoto M, Kimura K, Omata M, Ogawa T, Vallance P, Wilcox CS. Colocalization of demethylating enzymes and NOS and functional effects of methylarginines in rat kidney. *Kidney Int*. 1997;52:1593–1601.
- Terada Y, Tomita K, Nonoguchi H, Marumo F. Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylate cyclase messenger RNAs in microdissected rat nephron segments. *J Clin Invest*. 1992;90:659–665.
- Wilcox CS, Welch WJ. Macula densa nitric oxide synthase: expression, regulation, and function. *Kidney Int*. 1998;67(suppl):S53–S57.
- Mattson DL, Bellehumeur TG. Neural nitric oxide synthase in the renal medulla and blood pressure regulation. *Hypertension*. 1996;28:297–303.
- Mundel P, Bachmann S, Bader M, Fischer A, Kummer W, Mayer B, Kriz W. Expression of nitric oxide synthase in kidney macula densa cells. *Kidney Int*. 1992;42:1017–1019.
- Morrissey JJ, McCracken R, Kaneto H, Vehaskari M, Montani D, Klahr S. Location of an inducible nitric oxide synthase mRNA in the normal kidney. *Kidney Int*. 1994;45:998–1005.
- Haas JA, Khraibi AA, Perrella MA, Knox FG. Role of renal interstitial hydrostatic pressure in natriuresis of systemic nitric oxide inhibition. *Am J Physiol*. 1993;264:F411–F414.
- Khraibi AA. Inhibition of nitric oxide causes exaggerated natriuresis in spontaneously hypertensive rats. *Am J Physiol*. 1994;266:F762–F766.
- Khraibi AA. Role of renal nerves in natriuresis of L-NMMA infusion in SHR and WKY rats. *Am J Physiol*. 1995;269:F17–F21.
- Bagnall NM, Dent PC, Walkowska A, Sadowski J, Johns EJ. Nitric oxide inhibition and the impact on renal nerve-mediated antinatriuresis and antidiuresis in the anaesthetized rat. *J Physiol*. 2005;569:849–856.
- Morduchowicz GA, Sheikh-Hamad D, Jo OD, Nord EP, Lee DB, Yanagawa N. Increased Na<sup>+</sup>/H<sup>+</sup> antiport activity in the renal brush border membrane of SHR. *Kidney Int*. 1989;36:576–581.
- Gesek FA, Schoolwerth AC. Insulin increases Na<sup>+</sup>-H<sup>+</sup> exchange activity in proximal tubules from normotensive and hypertensive rats. *Am J Physiol*. 1991;260:F695–F703.
- Dagher G, Sauterey C. H<sup>+</sup> pump and Na<sup>+</sup>-H<sup>+</sup> exchange in isolated single proximal tubules of spontaneously hypertensive rats. *J Hypertens*. 1992;10:969–978.
- Lapointe MS, Sodhi C, Sahai A, Batlle D. Na<sup>+</sup>/H<sup>+</sup> exchange activity and NHE-3 expression in renal tubules from the spontaneously hypertensive rat. *Kidney Int*. 2002;62:157–165.
- Yip KP, Tse CM, McDonough AA, Marsh DJ. Redistribution of Na<sup>+</sup>/H<sup>+</sup> exchanger isoform NHE3 in proximal tubules induced by acute and chronic hypertension. *Am J Physiol*. 1998;275:F565–F575.
- Sneddon WB, Syme CA, Bisello A, Magyar CE, Rochdi MD, Parent JL, Weinman EJ, bou-Samra AB, Friedman PA. Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50). *J Biol Chem*. 2003;278:43787–43796.
- Yip KP, Tse CM, McDonough AA, Marsh DJ. Redistribution of Na<sup>+</sup>/H<sup>+</sup> exchanger isoform NHE3 in proximal tubules induced by acute and chronic hypertension. *Am J Physiol*. 1998;275:F565–F575.
- Wade JB, Liu J, Coleman RA, Cunningham R, Steplock DA, Lee-Kwon W, Pallone TL, Shenolikar S, Weinman EJ. Localization and interaction of NHERF isoforms in the renal proximal tubule of the mouse. *Am J Physiol Cell Physiol*. 2003;285:C1494–C1503.
- Weinman EJ, Steplock D, Wade JB, Shenolikar S. Ezrin binding domain-deficient NHERF attenuates cAMP-mediated inhibition of Na<sup>+</sup>/H<sup>+</sup> exchange in OK cells. *Am J Physiol Renal Physiol*. 2001;281:F374–F380.
- Kobayashi K, Monkawa T, Hayashi M, Saruta T. Expression of the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory protein family in genetically hypertensive rats. *J Hypertens*. 2004;22:1723–1730.
- Fukui T, Ishizaka N, Rajagopalan S, Laursen JB, Capers Q, Taylor WR, Harrison DG, de LH, Wilcox JN, Griendling KK. p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ Res*. 1997;80:45–51.
- Welch WJ, Baumgartl H, Lubbers D, Wilcox CS. Nephron pO<sub>2</sub> and renal oxygen usage in the hypertensive rat kidney. *Kidney Int*. 2001;59:230–237.
- Welch WJ, Tojo A, Wilcox CS. Roles of NO and oxygen radicals in tubuloglomerular feedback in SHR. *Am J Physiol Renal Physiol*. 2000;278:F769–F776.