NIH Public Access
Author Manuscript
Hypertension. Author manuscript; available in PMC 2010 December 1.

Published in final edited form as:
Hypertension. 2009 December ; 54(6): 1291–1297. doi:10.1161/HYPERTENSIONAHA.109.134783.

Renal proximal tubular reabsorption is reduced in adult spontaneously hypertensive rats: roles of superoxide and NHE3

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

Abstract

Proximal tubule reabsorption is regulated by systemic and intrinsic mechanisms, including locally produced autacoids. Superoxide (O_2^-), produced by NADPH oxidase (NOX) enhances NaCl transport in the loop of Henle and the collecting duct, but its role in the PT is unclear. We measured PT fluid reabsorption (J*v*) in WKY rats and compared that to J*v* in SHR, a model of enhanced renal O_2^- generation. Rats were treated with the NOX inhibitor apocynin (Apo), or with small interfering RNA (siRNA) for $p22^{phox}$, which is the critical subunit of NOX. J*v* was lower in SHR compared to WKY (WKY: 2.4±0.3 vs SHR: 1.1±0.2 nl/min/mm, n=9–11, p<0.001). Apo and siRNA to $p22^{phox}$ normalized J*v* in SHR yet had no effect in WKY. J*v* was reduced in proximal tubules perfused with S-1611, a highly selective inhibitor of the Na^+/H^+ exchanger 3 (NHE3), the major Na^+ uptake pathway in the proximal tubule, in WKY but not in SHR. Pretreatment with Apo restored an effect of S-1611 to reduce J*v* in the SHR (SHR+Apo: 2.9±0.4 vs SHR+Apo+S-1611: 1.0±0.3 nl/min/mm, p<0.001). However, since expression of NHE3 was similar between SHR and WKY, this suggests that O_2^- affects NHE3 activity. Direct microperfusion of tempol or apocynin into the PT also restored Jv in SHR. In conclusion, O_2^- generated by NOX, inhibits proximal tubule fluid reabsorption in SHR. This finding implies that PT fluid reabsorption is regulated by redox balance, which may have profound effects on ion and fluid homeostasis in the hypertensive kidney.

Keywords

Proximal reabsorption; superoxide; tempol; apocynin

Introduction

In the kidney, the proximal tubule reabsorbs 60–70% of filtered NaCl and fluid. Therefore changes in proximal tubule 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 proximal tubule is thought to mediate much of this pressure natriuresis response. In young spontaneously hypertensive rats (SHR), prior to the onset of hypertension, expression of the major Na^+ transport systems in the proximal tubules was higher [1] and Na^+ excretion was lower compared to normotensive rats (WKY) [2]. This was accompanied by an increase in fluid reabsorption in the proximal tubule in young (5 week) pre-hypertensive SHR compared to WKY. These observations suggest that an exaggerated NaCl and fluid reabsorption in the proximal tubule may contribute to the development of hypertension in young SHR, which persists in the adult

William J Welch, Ph.D., Georgetown University, Department of Medicine, 4000 Reservoir Road, Building D-395, Washington, D.C., 20057, welchw@georgetown.edu.

Conflict of interest disclosure: NONE.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

animal. However, the increased reabsorption seen in young animals is not consistently observed in adult SHR. For example, in 7 and 12 week old SHR, at a time when hypertension was established, baseline J*v* in the proximal tubule was lower compared to WKY[3]. J*v* was also lower in adult stroke-prone SHR[4], compared to WKY. This may be related to increased reactive oxygen species (ROS), since direct microperfusion of reducing agents corrected this dysfunction[4]. The SHR is indeed an established model of oxidative stress, with elevated superoxide levels and NADPH oxidase expression in both vascular and renal tissue [5].

The uncertain role of the proximal tubule is partially due to the difficult technologies to measure function. Proximal tubule transport in SHR has been investigated primarily *in vitro*, with cell preparations, or *in vivo* by free flow micropuncture. However, free flow measurements, which have characterized proximal tubule function on both WKY and SHR is complicated by interruption of flow to the macula densa, which tends to overestimate proximal tubule flow. Therefore, direct measurements of J*v* by *in situ microperfusion* is the most accurate method to characterize proximal tubule function, since this method separates transport from the confounding effects of flow and tubuloglomerular feedback (TGF). Therefore we measured J*v* in the proximal tubule of hypertensive and normotensive rats by direct microperfusion and recollection to test the hypothesis that increased production of O_2^- impairs proximal tubule function during hypertension in the adult SHR.

Materials and Methods

Animal Preparation

Groups of SHR and aged-matched WKY rats were pre-treated with apocynin (16 mg/kg/day), siRNA (25 μg, IV) or vehicle (IV) two days prior to 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, 80g/kg IP; Research Biochemicals, Inc, Natick, MA, USA) 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 blood pressure (MAP) (Powerlab, AD Instruments Inc, Colorado Spring, CO). 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, Birmingham Al) 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 NaCl and 1% albumin at 1.5 ml/h to maintain euvolemia [6]. Studies were begun after 60 minutes of stabilization.

Microperfusion of Proximal Tubules

The proximal tubule site was identified, as described previously[7], by injections from a "finding" pipette containing dye-stained artificial tubular fluid (ATF). The flow was blocked by injection of T grease (T grade, Apiezon Products, Manchester, UK) via a micropipette (10–12 μm OD) proximal to the perfusion site. The tubule was perfused with a micropipette (8–10 μm OD) connected to a microperfusion pump (model A1400, World Precision Instruments Inc, Sarasota, FL) at 18±3 nl/min. The perfusion solution contained ^{14}C inulin (New England Nuclear, Boston, MA) as volume marker and 0.1% FD&C green dye for identification of the perfused loops. Tubules were perfused for 2–8 minutes prior to fluid collections, which were made at a downstream site with a micropipette (7–10 μm OD) following placement of a column of oil to block downstream flow. Samples were collected for 3–5 minutes and transferred into a constant-bore capillary tube whose length was measured with a micrometer to calculate the tubular fluid volume [8]. Thereafter, the samples were injected into scintillation fluid and the ^{14}C activity counted. Collected samples with less than 95% and more than 105% of microperfused inulin were discarded. The amount of microperfused inulin was estimated by

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

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. Carver, MA). 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: $Jv = V_{perf}$ (nl/min)−V_{coll} (nl/min)/PT length (mm) and expressed nl/min/mm. The composition of the perfusion fluid was as follows in mM: 125 NaCl, 20 $NaHCO_3$, 5 KCl, 1 $MgSO_4$, 2 CaCl, 1 $NaHPO_4$, 5 glucose, and 4 urea. The *in vivo* nephron microperfusion technique allows control of flow rate, Na^+ concentration, osmolality, and extracellular pH. This method is limited by the lack of endogenous agents generated in the proximal tubule, since all perfusions are with artificial tubular fluid containing various antagonists, but it more directly measures the intrinsic transport properties of the epithelium and allows the use of various inhibitors to test the role of key players in regulation of proximal tubule 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 mid-proximal 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 3–4 minutes. The fluid was transferred to a capillary bore tube and measured by microscopic calibration.

Small interference RNA—The small interference RNA (siRNA) to rat $p22^{phox}$ (NM-024160) was validated *in-vitro* as previously reported [9]. The target site in $p22^{phox}$ cDNA of the constructs selected is: 299-320 (AAATTACTACGTCCGGGCTGT). The non-silencing control siRNA sequence ATTCTCCGAACGTGTCACGT (catalogue # 1022076; Qiagen, Valencia, CA) has no homology to any sequence in the mammalian genome. TransIT in-Vivo gene delivery system (Mirus, Madison, WI) was used to complex the $p22^{phox}$ siRNA to polymer as per manufacturer's recommendations. This complexed siRNA was brought to 6 ml volume and delivered via jugular vein in 7 seconds. Kidney cortex was harvested after 48 hrs and saved for gene analysis. In a separate group of SHR, proximal tubules were microdissected for measurement of mRNA.

Microdissection of Proximal tubules—The kidneys were perfused through the abdominal aorta with 10ml of cold dissection solution to rinse away the blood. The composition of the dissection solution was as follow, in mM: 135 NaCl, 5 KCl, 1 NaH2P04, 1.2 MgS04, 2 CaC12, 6 L-alanine, 10 hydroxyethylpiperazine-N′-2-ethanesulfonic acid, and 5.5 glucose, as well as 0.1% bovine serum albumin (BSA). The kidneys were then perfused with 10 ml of cold dissection solution containing 0.1% collagenase (CLS II, Worthington). Thin sagittal slices were cut from the perfused kidneys and incubated in dissection solution containing 0.1% collagenase at 37°C for 30 min during which the solution was bubbled with 100% oxygen. Microdissection of individual proximal tubule segments was performed in cold dissection solution under a stereomicroscope[10].

RNA Extraction, cDNA synthesis and Real time PCR—The kidney cortex and proximal tubules were harvested after 48 hrs and RNA was extracted using RNAqueous-4PCR kit (Ambion, Austin, TX). The cDNA was synthesized using superscript III with random hexamer (Invitrogen, Gaithersburg, MD). The gene expression for $p22^{phox}$ was assessed with real time PCR (ABI 7700, Foster City, CA), using FAM labeled $p22^{phox}$ Taqman probe assay (Rn00577357_m1, ABI, Foster City, CA) multiplexed with VIC labeled 18s control probe. Relative amounts of mRNA, normalizedby 18S rRNA, were calculated from threshold cycle numbers (C_T, i.e., $2^{-\Delta\Delta C_T}$).

SDS-PAGE gel electrophoresis and Western analysis—The kidney cortex was placed in RIPA lysis buffer containing protease inhibitors: PMSF 100 μg/ml, leupeptin 5 μg/

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

ml, aprotenin 5 μg/ml, and sodium fluoride 1mM/ml. The dissected sections with RIPA lysis buffer were homogenized in Fastprep Bio101 (Thermo, Milford, MA) and the tubes were spun at 12,500 rpm for 15 minutes in a cold centrifuge. The supernatant was aliquoted and frozen at −80°C for future analysis. Protein concentrations were determined by Bio-rad Protein Assay Reagent (Bio-rad, Hercules, CA). Protein lysate (100 μg) for each sample was denaturedin boiling water for 5 min. After denaturation the lysate was placed on ice for 5 minutes and loaded on to 12.5% SDS-PAGE gel (Bio-Rad, Hercules, CA). The gel was transferred to nitrocellulose membrane (Bio-rad, Hercules, CA). The membrane was blocked with 5% Blotto milk. The primary antibody for NHE3 2 μg/ml (ab22765) was from Abcam Inc., Cambridge, MA and the antibody for NHERF2 was the kind gift of Dr James B. Wade and Paul A. Welling (University of Maryland, Baltimore, MD). The secondary antibodies (1:10,000) were peroxidase-labeled goat anti-rabbit (KPL, Gaithersburg, MD). The blots were probed with Beta Actin (Sigma, St. Louis, MO) for equal loading. Densitometry for western analysis was performed with Image J program (NIH, Bethesda, MD) for NHE3 and NHERF2.

Statistical analysis: The effects of apocynin, siRNA to $p22^{phox}$, tempol and S-1611 in WKY and SHR were analyzed by 2 × 2 ANOVA, with post-hoc testing. Values are means ± SE. $P < 0.05$ was considered statistically significant.

Protocols

Protocol One

A. J*v* in the proximal tubule was measured in WKY and age-matched SHR, treated with vehicle (veh) or apocynin (Apo, an NADPH oxidase inhibitor, 16 mg/kg/day,) for 48 hours: Groups 1–4: WKY + veh; WKY + Apo; SHR + veh; SHR +Apo.

B. J*v* in the proximal tubule 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±6 mmHg (SHR +C, n=4).

C. Kidneys were harvested to measure protein expression of NHE3 and NHE regulatory factor-2 (NHERF2) in the renal cortex.

D. The acute effects of the superoxide dismutase (SOD) mimetic tempol and the antioxidant apocynin microperfused into the proximal tubule were tested in WKY and SHR.

Protocol Two—The effect of the inhibition of NHE3 was tested by direct microperfusion of S-1661 (10^{-5} M), a highly selective inhibitor to NHE3 in Groups 1–4 above.

Protocol Three—J*v* of the proximal tubule was measured in WKY and SHR treated with siRNA directed to $p22^{phox}$, a critical subunit of NADPH oxidase or scrambled siRNA (scr). Groups 5–8: WKY + scr; WKY + siRNA; SHR + scr; SHR + siRNA. Solutions (6 ml) containing siRNA were injected into cannulated jugular veins of anesthetized (1% isofluorane) rats. Animals were allowed to recover and prepared for microperfusion analysis after 48 hours.

Results

Mean arterial blood pressure (MAP), measured under anesthesia was higher in SHR compared to WKY (Table 1). Treatment for 2 days with Apo had no effect on MAP in either strain. Proximal flow (V_{PT}), measured by free flow collections in separate nephrons, was similar between WKY and SHR, but was decreased by Apo in SHR only ($p<0.001$). Distal flow (V_D) and urine flow were similar in all groups.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Protocol One

A. Absolute fluid reabsorption (J*v*) measured in the proximal tubule averaged 2.3±0.3 nl/min in normotensive WKY rats (Figure 1). However, J*v* was lower in proximal tubules of SHR (1.1±0.2 nl/min/mm, $p<0.001$). Apo, an inhibitor of NAPDH oxidase had no effect on J*v* in WKY, but restored J*v* to normal levels in SHR.

B. In a separate SHR group, renal perfusion pressure was maintained at 122±6 mmHg by an aortic clamp (SHR + C). J*v* was unaffected by the acute reduction in RPP (SHR: 1.1±0.2 vs SHR + C: 1.3±0.3 nl/min/mm, n=6–7, ns). This suggests the differences in J*v* between the two strains were not directly related to MAP.

C. Figure 2 shows that the protein expression of NHE3 in the renal cortex was similar in SHR compared to WKY. NHE3 in the renal cortex is exclusively expressed in the proximal tubule. NHE3 regulator factor-2 (NHERF2) is a PDZ domain binding element that is associated with reduced NHE-3 activity and translocation [11]. NHERF2 expression in the renal cortex was higher in SHR compared to WKY (Figure 2), suggesting that reduced NHE3 activity in SHR is due to overexpression of this regulatory factor. Indeed, NHERF-2 expression in SHR was reduced by Apo, further implicating this pathway in NHE3 dysregulation in SHR.

D. Figure 3 shows that direct microperfusion of tempol (10^{-4} M) had no effect in proximal tubules of WKY but normalized J*v* in proximal tubules of SHR (WKY: 2.3 ±0.5 vs WKY+tempol: 2.4±0.5 nl/min/mm, n=6–9 tubules, ns; SHR: 1.2±0.3 vs SHR +tempol: 2.4±0.5 nl/min/mm, n=7–10 tubules, $p<0.001$). Similarly, acute microperfusion of apocynin (10^{-5} M) also restored Jv in SHR (SHR: 1.2±0.3 vs SHR + APO: 2.0±0.3 nl/min/mm, $p<0.05$, n=8).

Protocol Two

To determine the mechanism of this dysfunction, we tested the activity of NHE3, a major Na^+ uptake path in the proximal tubule. J*v* was reduced by direct microperfusion of the NHE3 selective inhibitor, S-1611 (10^{-5} M, maximally effective dose) in WKY as expected, but not in SHR (Figure 4). This suggests that the dysfunction is due to reduction in NHE3 activity. When the reduced J*v* of SHR was restored by Apo, direct microperfusion of S-1611 lowered J*v*, similar to its effect in WKY (Figure 5). This suggests that the dysfunction of NHE-3 is linked to NAPDH oxidase generated O_2^-.

Protocol Three

To confirm that the reduction in J*v* of the proximal tubule was linked to NADPH oxidase, we repeated these experiments in rats treated with siRNA direct to $p22^{phox}$, the critical subunit of NADPH oxidase. mRNA expression of $p22^{phox}$, relative to expression of 18S, was reduced by siRNA treatment by 62±4% (n=6) in the renal cortical tissue and by 66±7% in microdissected proximal tubules (n=4) (Figure 6). J*v* in SHR was restored by siRNA to $p22^{phox}$, but unaffected in WKY (Figure 7). MAP was not affected by siRNA to $p22^{phox}$: (MAP scr siRNA: 118±3 vs MAP siRNA $p22^{phox}$: 115±3 mmHg, ns)

DISCUSSION

The major new finding of this study is that fluid reabsorption in the proximal tubule of adult SHR is impaired compared to WKY. Further, this reduced function can be completely restored by inhibition of O_2^-. Treatments that targeted O_2^- in general (tempol) and the more specific source of O_2^-, NADPH oxidase (Apo, siRNA) restored the lower J*v* of the PT in SHR. Two results suggest that these effects are independent of the high blood pressure in SHR. First, Apo and siRNA to $p22^{phox}$ did not lower BP in SHR, yet J*v* was corrected. Second, clamping of the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

renal artery in SHR 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 Na^+/H^+ exchanger-3 (NHE3). S-1611 reduced J*v* in WKY PT by 50%, but surprisingly had no effect on the reduced J*v* in SHR. 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. Since 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 SHR and WKY is linked to NHE3 activity, rather than content. NHE3 activity may depend on the NHE regulatory factors (NHERF). We showed that NHERF-2 expression was higher in SHR than WKY, which may account for reduced NHE3 activity in SHR. Further, Apo reduced NHERF2 expression, consistent with restoration of PT function. However, both Apo and tempol, microperfused acutely into the proximal tubule restored Jv in the SHR. This suggests that O_2^- also regulates J*v* directly and perhaps independently of NHE-3 and NHERF-2. Combined these results suggests that J*v* is regulated by a complex interaction of regulatory factors (NHERF-2), which translocates NHE-3 and more acutely by O_2^- inactivation of NHE-3. The acute effect of O_2^- may not be due to translocation of NHE3, but further studies will be required to determine this mechanism.

Young SHR have an enhanced proximal tubular reabsorption and sodium uptake[1], based on reports of greater NHE3 activity and lower 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 SHR[3]. These studies have popularized the concept that Na^+ retention in young SHR contributes to the development of hypertension. Yet this overactive PT does not persist into adulthood. The reduced J*v* in PT of SHR in this study contrasts with the findings in other studies that did not detect differences in PT flow or reabsorption between adult SHR and WKY[12]. In fact, many studies do not report differences in Na^+ excretion or transporter activities between adult SHR and normotensive rats. However, direct measurements have shown that J*v* is lower in adult hypertensive rats compared to normotensive rats [4]. Therefore, our observations with microperfusion and recollection in the SHR confirm these earlier studies that used the split-drop methodology. Further, Wu and Johns showed the reduced J*v* could be restored by local delivery of exogenous superoxide dismutase (SOD)[4]. We confirmed this observation using the membrane-permeant antioxidant tempol and apocynin. Further, we have extended the studies on the role of O_2^- in regulation of proximal tubule function by NHE3 and NHERF2 expression.

As noted above, PT flow was similar in SHR compared to normotensive rats[12]. Indeed we also failed to see differences in PT flow between WKY and SHR when measured by free flow collections in separate nephrons (Table 1). However, when O_2^- 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 due to the method used in this study, which blocks flow to the macula densa and tends to increase GFR and therefore, PT flows in both strains. Additionally, similar flow in this segment could be real and the differences in transport function between these strains occurs in segments not evaluated in this study.

How is the PT regulated by O_2^-? One possibility is the interaction of O_2^- and nitric oxide (NO). NO inhibits Na^+ uptake in the TALH[13] via its effects on cGMP and on the density of membrane channels. However, NO *promotes* Na^+ and/or fluid reabsorption in the proximal tubule[14]. J*v*, measured by the same method in this study was lower in iNOS and nNOS knockout mice[15, 16]. J*v* in normal rat proximal tubule is also reduced by NOS inhibition[17]. Taken together, these results suggest that endogenous NO enhances fluid transport in the proximal tubule [18]. Therefore O_2^-, which reduces bioavailable NO should inhibit fluid reabsorption in

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

the proximal tubule, which is consistent with our finding. However, NOS expression in the proximal tubule is not consistently shown, therefore the source of NO is not known. Yet there is considerable evidence that proximal tubule is constantly exposed to NO, since NOS is expressed in adjacent tissue and cells. In the kidney eNOS is expressed predominantly in the renal vascular endothelial cells [19, 20]. Also nNOS is expressed in the epithelium, Bowman's capsule and especially in the macula densa [21–24], which is in close contact with the proximal tubule in the renal cortex. iNOS is widely expressed in the tubule epithelia, including the proximal tubule, the thick ascending limb of Henle's loop, and distal convolute tubule [19, 24]. Data supporting the effects of NO to enhance Na^+ uptake is difficult to obtain, since systemic administration of NOS inhibitors increases BP and renal perfusion pressure [25–27]. However, an acute dose of an iNOS inhibitor, but not an nNOS inhibitor, both of which did not increase MAP, increased Na^+ excretion 2-fold in rats[28]. Also, we showed that microperfusion of the proximal tubule with a non-selective NOS inhibitor reduced J*v* by 30–40%, suggesting that NO promotes Na^+ uptake in the proximal tubule[17].

Another possibility suggested by our data is that O_2^- inhibits NHE3 activity. NHE3 is a major Na^+ uptake mechanism in the proximal tubule and increased activity of NHE3 in the renal brush border membrane (BBM) may be involved in the pathogenesis of hypertension [29–32]. However, in elegant experiments, McDonough and colleagues show that NHE3 is inactivated by translocation [33, 34]. Therefore reduced reabsorption in proximal tubule of SHR may be due to a relocation of the NHE3, which would alter function [34, 35]. 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_2^- lead to translocation of NHE3 and antioxidants reactivate this pathway. Further, the regulatory factor for NHE3, NHERF2 may participate in the redox regulation of NHE3. Increased NHERF2 leads to translocation and inactivation of NHE3[36, 37]. We confirmed that NHERF2 is higher in SHR [38]. This increase is due to higher levels of O_2^-, since Apo reduced NHERF2 levels and increased NHE3 expression in the renal cortex and improved Jv in the PT in the SHR.

Renal levels of O_2^- and NADPH oxidase (NOX) are higher in SHR compared to WKY [39, 40]. In addition, in this study we show that $p22^{phox}$ mRNA is expressed in microdissected proximal tubules and is reduced by systemic infusion of siRNA to $p22^{phox}$. Also, direct microperfusion of tempol and apocynin into the proximal tubule suggests that the increased O_2^- in the SHR is derived from NOX in the proximal tubule. Consistent with this view, we showed that the SHR kidneys also had greater expression of NO synthase, yet bioavailable NO was lower in SHR[41]. Treatment with tempol increased NO function and presumably NO levels, indicating the powerful scavenging effects of O_2^-.

Further explanation of these results is required, since Na^+ and fluid excretion of age-matched SHR and WKY are similar[12]. We suggest that downstream sites of Na^+ reabsorption are increased by O_2^-, which compensates for the dysfunction in the PT. Garvin and colleagues have shown that O_2^- increased Na^+ reabsorption in the TALH and the collecting duct, which could normalize excretion. The shift in nephron relative function can potentially alter oxygen consumption, since the PT is more O_2 efficient for Na^+ transport than downstream segments. Therefore, the increased O_2^- in SHR may contribute to the reduced oxygen efficiency and renal hypoxia seen in this strain[40].

In conclusion, fluid uptake in the proximal tubule of adult SHR is impaired due to increased NADPH oxidase-dependent O_2^-. Our data suggests that O_2^- reduces NHE3 activity, possibly due to increased expression of NHE3 regulatory factor, but also inhibits fluid uptake independently of these pathways. These data suggest that increased ROS alters proximal tubular reabsorption during hypertension and impacts renal regulation of fluid and solute balance.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Perspectives

These data suggest that during hypertension responsibility for Na^+ and fluid reabsorption along the nephron changes. Reabsorption in the proximal tubule is reduced and is increased downstream. This has an impact on oxygen usage for Na^+ uptake since the PT is relatively more efficient than more distal segments. Therefore to preserve normal Na^+ excretion, the kidney burns more oxygen as it transfers work during chronic increase in blood pressure. This may be a normal adaptation as the kidney acts to maintain stable Na^+ balance. Increased superoxide 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.

Acknowledgments

Source of funding

The studies reported in this manuscript were support by the National Institutes of Health (NIH) grants HL089583 and DK072183.

References

1. Aldred KL, Harris PJ, Eitle E. Increased proximal tubule NHE-3 and H+-ATPase activities in spontaneously hypertensive rats. J Hypertens 2000:623–628. [PubMed: 10826566]
2. 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 2005:1805–1815. [PubMed: 15698858]
3. Thomas D, Harris PJ, Morgan TO. Age-related changes in angiotensin II-stimulated proximal tubule fluid reabsorption in the spontaneously hypertensive rat. J Hypertens Suppl 1988:S449–S451. [PubMed: 2853752]
4. Wu XC, Johns EJ. Interactions between nitric oxide and superoxide on the neural regulation of proximal fluid reabsorption in hypertensive rats 18. Exp Physiol 2004:255–261. [PubMed: 15123560]
5. 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 February;:269–274. [PubMed: 11847196]
6. Welch WJ, Wilcox CS. Potentiation of tubuloglomerular feedback in the rat by thromboxane mimetic. Role of macula densa. J Clin Invest 1992:1857–1865. [PubMed: 1601993]
7. 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:F37–F41. [PubMed: 18480183]
8. Wilcox CS, Welch WJ, Schreiner GF, Belardinelli L. Natriuretic and diuretic actions of a highly selective adenosine A1 receptor antagonist. J Am Soc Nephrol 1999:714–720. [PubMed: 10203354]
9. 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 2006:238–244. [PubMed: 16391171]
10. Tojo A, Tisher CC, Madsen KM. Angiotensin II regulates H+-ATPase activity in rat cortical collecting duct. Am J Physiol (Renal Fluid Electrolyte Physiol) 1994:F1045–1051.
11. 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:C192–C198. [PubMed: 11121391]
12. Arendshorst WJ, Beierwaltes WH. Renal tubular reabsorption in spontaneously hypertensive rats. Am J Physiol 1979:F38–F47. [PubMed: 464059]
13. Ortiz PA, Garvin JL. Superoxide stimulates NaCl absorption by the thick ascending limb. Am J Physiol Renal Physiol 2002:F957–F962. [PubMed: 12372771]
14. Wang T. Nitric oxide regulates. Am J Physiol 1997:F242–F248. [PubMed: 9124402]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

15. Wang T, Inglis FM, Kalb RG. Defective fluid and HCO(3)(−) absorption in proximal tubule of neuronal nitric oxide synthase-knockout mice. Am J Physiol Renal Physiol 2000:F518–F524. [PubMed: 10966931]
16. Wang T. Role of iNOS and eNOS in modulating proximal tubule transport and acid-base balance. Am J Physiol Renal Physiol 2002:F658–F662. [PubMed: 12217856]
17. 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. J Am Soc Nephrol Abstract 2008:19–23A.
18. Wang T, Inglis FM, Kalb RG. Defective fluid and HCO(3)(−) absorption in proximal tubule of neuronal nitric oxide synthase-knockout mice. Am J Physiol Renal Physiol 2000:F518–F524. [PubMed: 10966931]
19. 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 1997:1593–1601. [PubMed: 9407505]
20. 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 1992:659–665. [PubMed: 1379616]
21. Wilcox CS, Welch WJ. Macula densa nitric oxide synthase: expression, regulation, and function. Kidney Int Suppl 1998:S53–S57. [PubMed: 9736254]
22. Mattson DL, Bellehumeur TG. Neural nitric oxide synthase in the renal medulla and blood pressure regulation. Hypertension 1996:297–303. [PubMed: 8707397]
23. 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 1992:1017–1019. [PubMed: 1280698]
24. 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 1994:998–1005. [PubMed: 7516453]
25. 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:F411–F414. [PubMed: 8456954]
26. Khraibi AA. Inhibition of nitric oxide causes exaggerated natriuresis in spontaneously hypertensive rats. Am J Physiol 1994:F762–F766. [PubMed: 8203560]
27. Khraibi AA. Role of renal nerves in natriuresis of L-NMMA infusion in SHR and WKY rats. Am J Physiol 1995:F17–F21. [PubMed: 7631827]
28. 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 2005:849–856. [PubMed: 16239274]
29. Morduchowicz GA, Sheikh-Hamad D, Jo OD, Nord EP, Lee DB, Yanagawa N. Increased Na+/H+ antiport activity in the renal brush border membrane of SHR. Kidney Int 1989:576–581. [PubMed: 2554051]
30. Gesek FA, Schoolwerth AC. Insulin increases Na(+)-H+ exchange activity in proximal tubules from normotensive and hypertensive rats. Am J Physiol 1991:F695–F703. [PubMed: 1674643]
31. Dagher G, Sauterey C. H+ pump and Na(+)-H+ exchange in isolated single proximal tubules of spontaneously hypertensive rats. J Hypertens 1992:969–978. [PubMed: 1328378]
32. Lapointe MS, Sodhi C, Sahai A, Batlle D. Na+/H+ exchange activity and NHE-3 expression in renal tubules from the spontaneously hypertensive rat. Kidney Int 2002:157–165. [PubMed: 12081574]
33. Yip KP, Tse CM, McDonough AA, Marsh DJ. Redistribution of Na+/H+ exchanger isoform NHE3 in proximal tubules induced by acute and chronic hypertension. Am J Physiol 1998:F565–F575. [PubMed: 9755128]
34. 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) 25. J Biol Chem 2003:43787–43796. [PubMed: 12920119]
35. Yip KP, Tse CM, McDonough AA, Marsh DJ. Redistribution of Na+/H+ exchanger isoform NHE3 in proximal tubules induced by acute and chronic hypertension. Am J Physiol 1998:F565–F575. [PubMed: 9755128]
36. 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 26. Am J Physiol Cell Physiol 2003:C1494–C1503. [PubMed: 12917102]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

37. Weinman EJ, Steplock D, Wade JB, Shenolikar S. Ezrin binding domain-deficient NHERF attenuates cAMP-mediated inhibition of Na(+)/H(+) exchange in OK cells 40. Am J Physiol Renal Physiol 2001:F374–F380. [PubMed: 11457730]

38. Kobayashi K, Monkawa T, Hayashi M, Saruta T. Expression of the Na+/H+ exchanger regulatory protein family in genetically hypertensive rats. J Hypertens 2004:1723–1730. [PubMed: 15311100]

39. 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 1997:45–51. [PubMed: 8978321]

40. Welch WJ, Baumgartl H, Lubbers D, Wilcox CS. Nephron pO2 and renal oxygen usage in the hypertensive rat kidney. Kidney Int 2001:230–237. [PubMed: 11135075]

41. Welch WJ, Tojo A, Wilcox CS. Roles of NO and oxygen radicals in tubuloglomerular feedback in SHR. Am J Physiol Renal Physiol 2000:F769–F776. [PubMed: 10807588]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

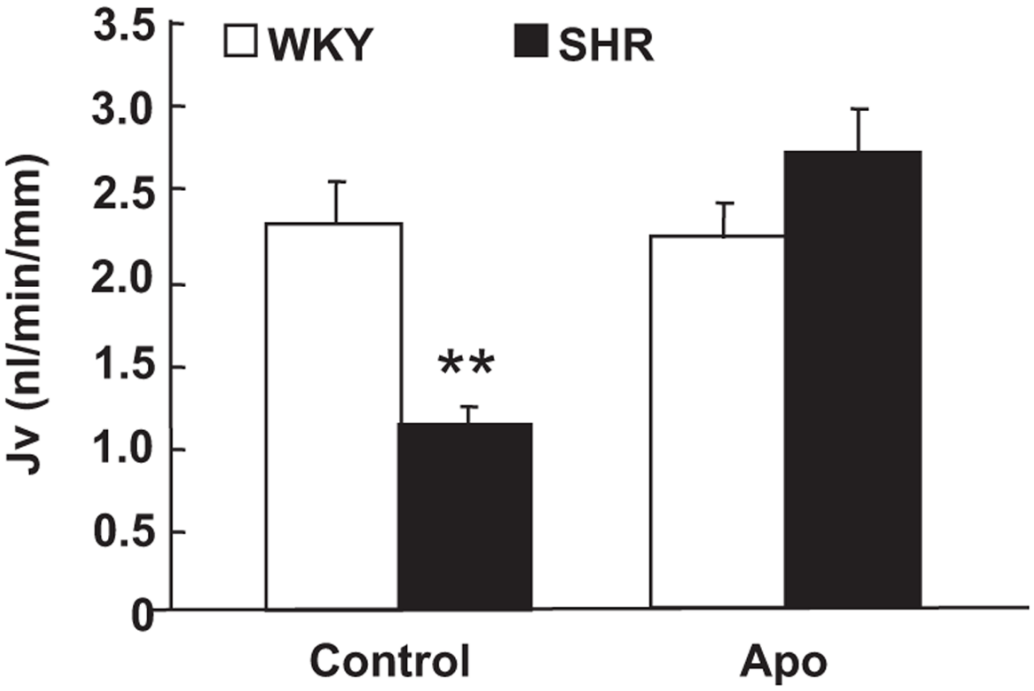

Figure 1.
Absolute fluid absorption (*Jv*) in PT of WKY (n=9–11) and SHR (n=9–11): effects of systemic apocynin (Apo, 16 mg/kg/day for 2 days).
**, $p<0.001$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

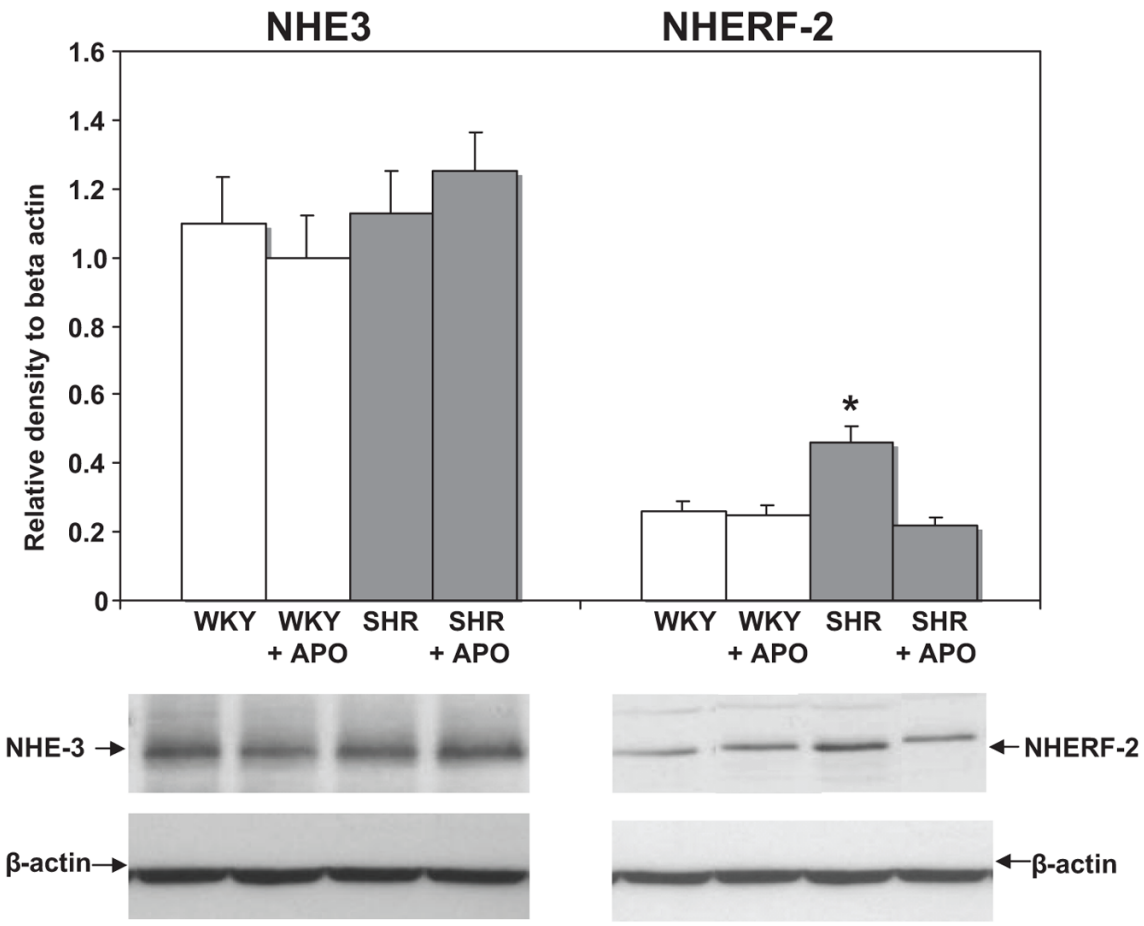

Figure 2.
Effect of apocynin (2 days) on NHE3 and NHERF2 expression in the renal cortex of WKY and SHR.
*, $p<0.01$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

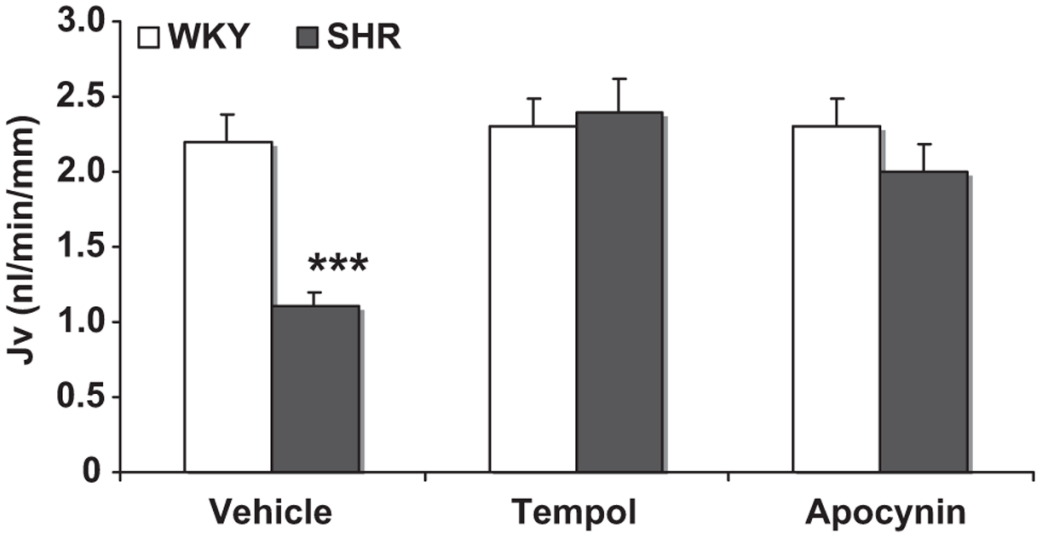

Figure 3.
Effect of microperfusion of tempol (10^{-4} M) or apocynin (10^{-5} M) directly into PT on J*v* in SHR and WKY (n=7–9).
**, p<0.001.

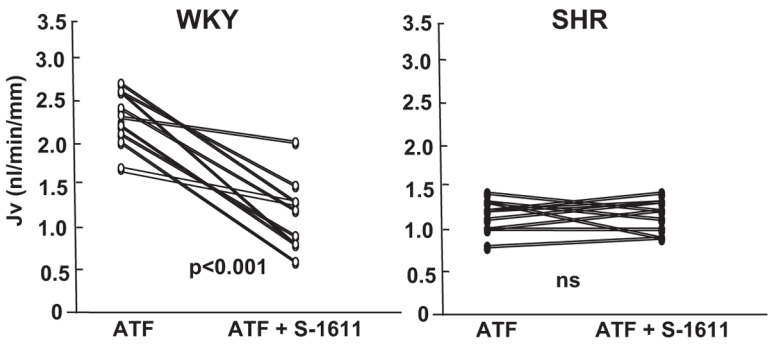

Figure 4.
J*v* in PT of WKY (n=8) and SHR (n=7): effects of PT microperfusion of NHE3 inhibitor, S-1611.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

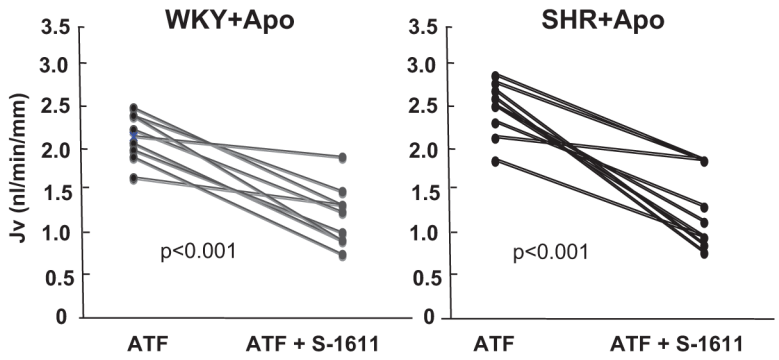

Figure 5.
J*v* in WKY (n=9) and SHR (n=7) after Apo: effects of microperfusion of NHE3 inhibitor, S-1611.

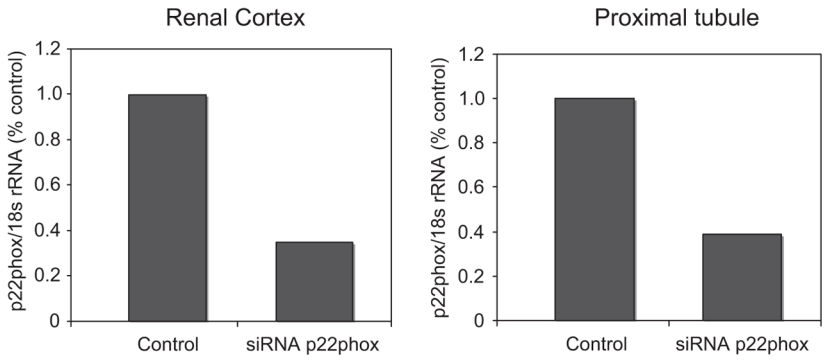

Figure 6.

p22^{phox} mRNA expression in renal cortex and in microdissected proximal tubules: effects of systemic injection of siRNA directed to p22^{phox} 48 hours prior to tissue collection (n=3–6).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

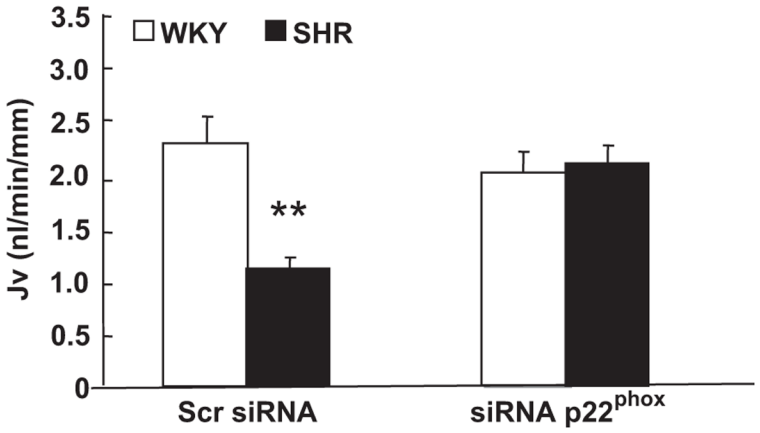

Figure 7.

Jv in PT of WKY (n=6) and SHR (n=9): effects of siRNA to $p22^{phox}$ (systemic injection 48 hours prior to experimentation).

**p<0.001.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Table 1

Mean arterial pressure and renal parameters

	MAP (mmHg)	GFR (ml/min)	VPT (nl/min)	VDT (nl/min)	UV (μl/min)
WKY (n=6–10)	100±5	1.9±0.3	13.9±0.9	6.5±1.2	4.6±0.2
SHR (n=6–9)	185±6*	1.7±0.5	12.2±0.9	5.9±0.8	4.4±0.5
WKY+APO (n=4–7)	95±3	2.3±0.6	13.3±0.5	5.5±0.8	4.0±0.3
SHR +APO (n=4–7)	190±6*	2.0±0.5	8.3±0.5*	5.3±0.3	4.5±0.5

MAP, mean arterial blood pressure, GFR, glomerular filtration rate, V_{PT}, proximal tubule flow, V_D, distal tubule flow, U_V, urine flow.

Each parameter analyzed by 2 × 2 ANOVA.

* $p<0.001$.