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Effect of angiotensin-II on renal Na^+/H^+ exchanger-NHE3 and NHE2

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Abstract

The purpose of the present study was to determine the effect of angiotensin II (A-II) on membrane expression of Na^+/H^+ exchange isoforms NHE3 and NHE2 in the rat renal cortex. A-II (500 ng/kg per min) was chronically infused into the Sprague–Dawley rats by miniosmotic pump for 7 days. Arterial pressure and circulating plasma A-II level were significantly increased in A-II rats as compared to control rats. pH-dependent uptake of $^{22}Na^+$ study in the presence of 50 μM HOE-694 revealed that Na^+ uptake mediated by NHE3 was increased ~ 88% in the brush border membrane from renal cortex of A-II-treated rats. Western blotting showed that A-II increased NHE3 immunoreactive protein levels in the brush border membrane of the proximal tubules by 31%. Northern blotting revealed that A-II increased NHE3 mRNA abundance in the renal cortex by 42%. A-II treatment did not alter brush border NHE2 protein abundance in the renal proximal tubules. In conclusion, chronic A-II treatment increases NHE3-mediated Na^+ uptake by stimulating NHE3 mRNA and protein content.

Keywords: Na^+/H^+ exchange; Proximal tubule; Kidney; Sodium uptake

1. Introduction

The sodium-hydrogen exchangers (NHEs) are plasma membrane-bound proteins that mediate the movement of extracellular Na^+ into cells in exchange for intracellular H^+. Eight NHE isoforms have been identified from mammalian cells [1]. Among them, five NHE isoforms (NHE1, NHE2, NHE3, NHE4 and NHE8) have been described in the kidney. NHE1 and NHE4 are located in the basolateral membrane of the renal tubule, and NHE2, NHE3 and NHE8 are apical isoforms [1–3]. Specifically, NHE3 is expressed in S1 and S2 segments of the proximal tubule and in the cortical thick ascending limb of the loop of Henle [4–8]. NHE3 plays a significant role in Na^+ and fluid reabsorption in the proximal tubule. NHE3 null mice have reduced Na^+ and HCO_3^- reabsorption in the proximal tubule with urinary Na^+ and HCO_3^- wasting [9].

Angiotensin II (A-II) is an essential hormone that exerts pleiotropic actions in the renal proximal tubule including modulation of transport [10,11], metabolism [12], and cell proliferation [13]. A-II is a powerful vasoconstrictor and strong mediator of intravascular volume regulation [14]. A-II acts indirectly on the distal nephron through aldosterone secretion by the adrenal glands, but it also acts directly on the proximal tubule in regulating NaCl reabsorption [14]. The renal proximal tubule is the main site for renal water, salt, and HCO_3 resorption [5]. It is well established that A-II increases renal Na^+ and HCO_3 reabsorption [15,16]. Studies from micropuncture and microperfusion also indicated that proximal tubule Na^+ and water transport on the peritubular side was stimulated by physiological concentrations of A-II [17]. Moreover, studies have shown that A-II stimulates Na^+ uptake in isolated proximal tubule cells through the amiloride-sensitive Na^+/H^+ exchanger, and this activation can be inhibited by the receptor antagonist saralasin [16]. Recently, study have shown that increased abundance of NHE3 in thick ascending limb cells as well as in proximal tubule brush border may contribute to enhanced renal Na^+ and HCO_3 reabsorption in response to A-II [18].

NHE2 and NHE3 have a more restricted tissue distribution, being predominantly located in the apical membrane of epithelia, and are highly expressed in the intestine and in the kidney. They are thought to be involved in bicarbonate, and water reabsorption [19]. However, the

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relative roles of these two isoforms in these processes are not completely understood.

Until now, the effect of A-II on NHE2-mediated Na^+ reabsorption in the kidney remains unknown. To understand the effect of A-II on renal sodium transport mediated by NHE3 and NHE2 in the proximal tubule, we tested the A-II infusion model and used this model to investigate molecular mechanisms of prolonged A-II administration on renal NHE3 and NHE2 expression. Our result showed that prolonged A-II treatment increased apical NHE3-mediated Na^+ absorption, apical NHE3 protein and NHE3 mRNA abundance. However, apical NHE2 transporter in the renal cortex was not affected by A-II treatment based on Western blotting analysis. The current findings suggest that the increase in apical NHE3 activity is a reflection of increased NHE3 gene expression in response to A-II.

2. Materials and methods

2.1. Preparation of animals and tissues

Adult male Sprague–Dawley rats (Harlan, Madison, WI) were housed separately in overhanging cages and maintained in a temperature, light and humidity controlled room. Throughout the experiments, animals had free access to water and standard rat chow (Ralson Purina, St. Louis, MO). All animal work was approved by the University of Arizona Institutional Animal Care and Use Committee. Rats (weighing 280–300 g; $n=28$) were anesthetized with sodium pentobarbital (50 mg/kg body wt; administered intraperitoneally), and an osmotic minipump (model 2001; Alza, Palo Alto, CA) was implanted subcutaneously at the dorsum of the neck. Rats were randomly assigned to two experimental groups. One group of 16 rats received A-II infusion (Sigma, St. Louis, MO), at a rate of 130 ng/min (approximately 500 ng/kg per min), for a period of 7 days, while another group of 12 rats received saline and served as controls. Preliminary studies with 1, 2, 3 and 7 days of infusions were carried out to determine duration of A-II infusion.

Several rats were maintained in metabolic cages, and 24-h urine and water intake were collected 1 day prior to implantation of minipumps to establish the baseline value and on days 4 and 7 of infusion. The concentrations of sodium and creatinine in urine samples were measured by University of Arizona Animal Care Diagnostic Laboratory.

Systolic arterial BP was measured in conscious rats on day 6 of infusion, using tail-cuff plethysmography (IITC INC/Life Science Instruments, Model 179; Woodland Hills, CA). Rats were killed by CO_2 anesthesia followed by cervical dislocation. Blood samples were collected into chilled tubes containing EDTA. Plasma was separated and stored at -80 °C until a plasma A-II concentration assay was performed with an enzyme immunoassay kit (Spibio, France). One renal cortex was excised, snap-frozen in liquid nitrogen, and stored at -80 °C until processing for mRNA purification. The other renal cortex was used for brush border membrane vesicle (BBMV) preparation on the day of BBMV uptake or for Western blot analyses and was never frozen.

2.2. BBMV preparation of rat renal cortex

BBMV were prepared from the renal cortex by the $MgCl_2$ precipitation technique as previously described [20,21].

2.3. Uptake analysis of rat renal BBMV

pH-dependent uptake of radiolabeled sodium was measured by a rapid filtration technique as previously described [20,22]. Protein was quantitated by a Bradford protein assay. The final BBMV pellets were resuspended in either preincubation buffer for no pH gradient condition [intracellular pH (pH_i) and extracellular pH (pH_0) equal 7.5] [100 mM TMA-gluconate, 85 mM HEPES, 45mM Tris/HCl (pH 7.5)], or preincubation buffer with an outwardly directed pH gradient (pH_i 5.2, pH_0 7.5) [100 mM TMA-gluconate, 90 mM MES, 40 mM HEPES/Tris (pH 5.2)], and placed at 25 °C for 1 h. Briefly, transport was initiated by adding 20 μl of the final BBMV suspension to 80 μl incubation solution [same as preincubation buffer (pH 7.5)], with the addition of 31.25 μCi $^{22}Na^+$/10 ml. Studies were performed in the presence of 50 μM HOE-694, a specific NHE inhibitor, which allows selective inhibition of NHE2 [inhibition constant (K_i) = 5 μM in PS120 cells], without affecting NHE3 (K_i = 650 μM in PS120 cells) [23]. HOE-694 [(3-methylsulfonyl-4-piperidinobenzoyl) guanidine methanesulfonate] was kindly provided by Dr. H.J. Lang (Hoechst Marion Roussel Pharmaceuticals, Frankfurt am Main, Germany). The reactions were stopped after 10 s by adding 2 ml ice-cold stop solution (185 mM potassium-gluconate, 10 mM Tris, 16 mM HEPES, 0.1 mM amiloride). The vesicles were immediately collected on a cellulose nitrate filter (0.45 μm pore size) and kept under suction while they were washed with 5 ml ice-cold stop solution. The amount of radioactive substrate remaining on the filter was determined in a Beckman liquid scintillation counter, with ReadySafe (Beckman, Fullerton, CA) as the liquid scintillant. Radioactivity remaining on the filters after pipetting incubation medium into the radioactive substrate in the absence of vesicles was used as background, and was considered in all calculations. Uptake values were determined by subtracting the uptake levels with no pH gradient condition (pH_i/pH_0 = 7.5/7.5) from those with an outwardly directed pH gradient (pH_i/pH_0 = 5.2/7.5). All values are expressed as pmol of sodium uptake per mg of vesicular protein in 10 s. Three repetitions were performed on membrane vesicle preparations from three different groups of animals from both A-II-infused and saline-infused rats and at least three rats for each group.

2.4. Western blot analysis of rat renal BBMV with NHE2- and NHE3-specific antiserum

Protein (20 μg) was placed in a twofold excess of Laemmli solubilization buffer plus 2 mM β-mercaptoethanol (β-ME), boiled for 5 min and placed on ice. Protein samples were fractionated by 7.5% SDS polyacrylamide gel electrophoresis (SDS/PAGE), and transferred onto nitrocellulose membranes. Blots were reacted with 1:4000 dilutions of rabbit NHE3-specific polyclonal antibody as previously described [24,25]. This antibody was raised against a fusion protein encompassing amino acids 699–831 from the COOH-terminal portion of the NHE3 protein generated by baculovirus-mediated expression in Sf9 cells [26]. Additionally, some blots were reacted with 1:500 dilutions of NHE2-specific antiserum as previously described [26]. The anti-mouse/anti-rabbit secondary antibody (40 mU/ml; Boehringer Mannheim GmbH, Germany) was used with the reagent for chemiluminescent detection (Boehringer Mannheim). Membranes were stripped and subsequently reacted with β-actin antiserum (Sigma) at 1:5000 dilution. NHE3- or NHE2-specific band intensities were determined by densitometric analysis [utilizing GS-700 Imaging Densitometer and Quantity One software (Bio-Rad, Hercules, CA)] and were normalized for β-actin band intensities on the same blot. Experiments were repeated three times with protein samples isolated from different groups of animals from both A-II-infused and saline-infused rats.

2.5. RNA purification and Northern blot analyses of rat renal cortex

mRNA was isolated from the renal cortex, utilizing the Fast-Track mRNA purification kit (Invitrogen, Carlsbad, CA). Northern blots were carried out as previously described, using 5 μg mRNA/lane [24,25]. Rat NHE3-specific cDNA radiolabeled probes (*Dra*I- and *Kpn*I-digested fragment) [24] were used for hybridization. High-stringency washes were performed at 65 °C with 0.1× SSC–0.1% SDS. Northern blots were subsequently stripped and reprobed with 1B15-specific cDNA probes (1B15 encodes rat cyclophilin [2]), which were used as internal standards for quantitating NHE3 mRNA expression. Blots were exposed to phosphor imaging screens, and band intensities were determined with Quantity One Software (FX Molecular Imager; Bio-Rad). Experiments were repeated three times with mRNA samples isolated from different groups of animals from both A-II-infused and saline-infused rats.

2.6. Statistical analyses

The experimental data are expressed as means ± S.E. They were analyzed by ANOVA (StatView 5.0.1 version; SAS Institute, Cary, NC). P values of <0.05 indicate statistical significance.

3. Results

3.1. Blood pressure and plasma A-II levels

Blood pressure and plasma A-II levels were measured in some rats in this current study. On day 6 of infusion, systolic BP was significantly elevated in the A-II-treated rats, compared to controls (190 ± 4 mm Hg in A-II treated rats, versus 120 ± 3 mm Hg in control rats; $n=6$ rats, $P<0.001$).

On day 7 of infusion, circulating plasma A-II levels were markedly increased in the A-II group, compared to controls (53.0 ± 7.7 fmol/ml plasma in A-II treated rats, versus 8.1 ± 1.5 fmol/ml plasma in control rats; $n=5$ rats, $P<0.02$).

3.2. Water intake, urine output and Na^+ excretion

Water intake for the two groups was identical at the initiation of the study (A-II, 35.0 ± 2.2 ml/day, $n=6$ rats; control, 40.0 ± 6.3 ml/day, $n=5$ rats, $P=0.4$). On day 4 of

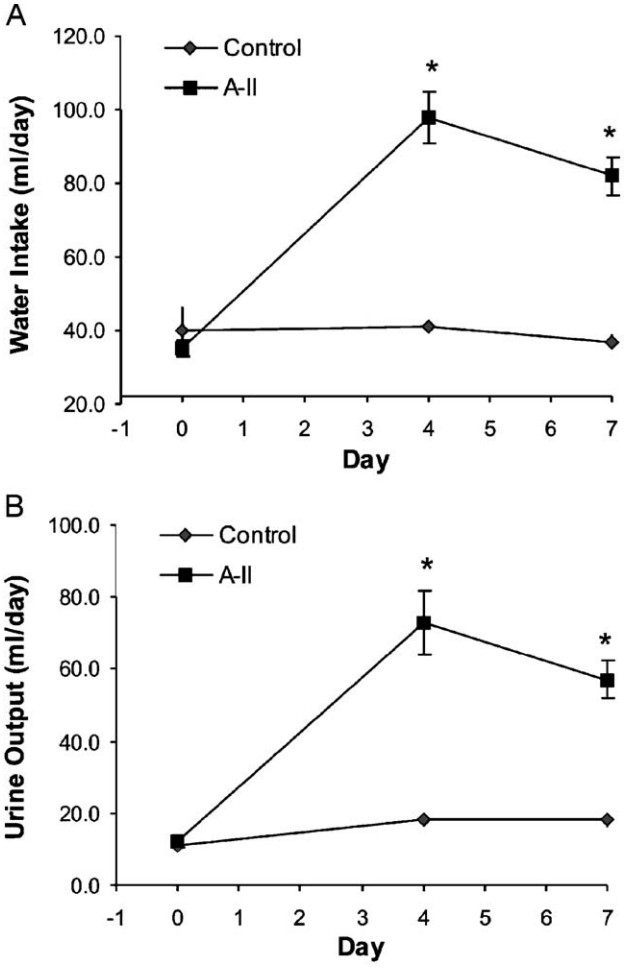

Fig. 1. Effect of A-II infusion on water intake (A), urine output (B). Rats were infused with saline or A-II (500 ng/kg per min) by osmotic minipump for 7 days. Water intake, urine output were measured 1 day before pump implantation, at 4 and 7 days of infusion. Values are mean ± S.E. of six rats.

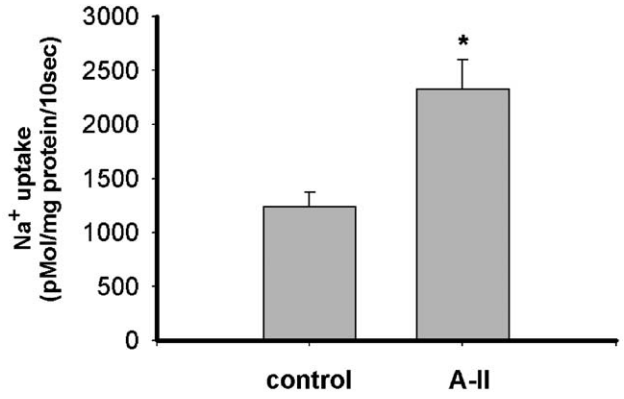

Fig. 2. Na^+ uptake analysis of rat renal cortical BBMV. BBM vesicles were prepared from groups of rats treated with A-II or saline. pH-dependent uptake of $^{22}Na^+$ were performed in the presence of 50 μM HOE-694. Values are mean ± S.E.; number of replicates = 3. *$P < 0.05$.

infusion, the average water intake for the A-II-treated rats was significantly higher than that for the saline-treated rats (98.0 ± 7.0 ml/day in A-II group, versus 41.0 ± 1.5 ml/day in controls, $P = 0.0001$). On day 7 of infusion, the average water intake for the A-II-treated rats was significantly higher than that for the saline-treated ones (82.0 ± 5.0 ml/day in A-II group, versus 37.0 ± 1.9 ml/day in controls; $P = 0.0001$) (Fig. 1A).

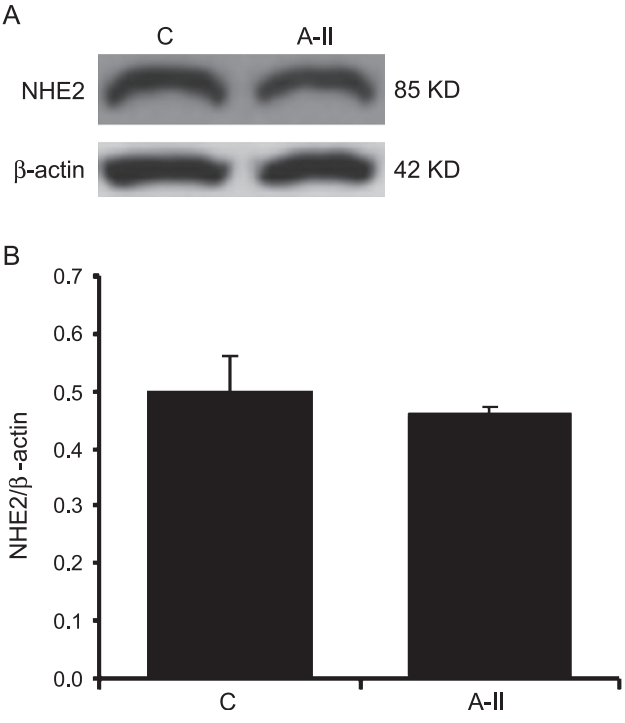

Fig. 3. Western blot analysis of NHE2 protein in rat renal cortical BBMV. Panel A shows one typical Western blot experiment. Western blots were reacted with NHE2-specific and β-actin-specific antiserum. The band at 85 kDa is NHE2 and the band at 42 kDa is β-actin. Panel B shows quantitative data from NHE2 Western blot experiments. Data are presented as a ratio of NHE2 to β-actin protein levels. Values are mean ± S.E.; number of replicates = 3.

Urine output for the two groups was identical at the initiation of the study (A-II, 12.0 ± 1.6 ml/day, $n = 6$ rats; control, 11.0 ± 1.0 ml/day, $n = 5$ rats, $P = 0.6$). On day 4 of infusion, the average urine output for the A-II treated rats was significantly higher than that for the saline-treated ones (73.0 ± 8.7 ml/day in A-II group, versus 18.0 ± 1.3 ml/day in controls, $P = 0.0003$). On day 7 of infusion, the average urine output for the A-II-treated rats was significantly higher than that for the saline-treated ones (57.0 ± 5.2 ml/day in A-II group, versus 18.0 ± 1.3 ml/day in controls, $P = 0.0001$) (Fig. 1B).

Urine sodium excretion was normalized to urine creatinine concentration. Urine sodium/creatinine ratio for the two groups was identical at the initiation of the study (A-II, 3.1 ± 0.2, $n = 6$ rats; control, 2.6 ± 0.4, $n = 5$ rats, $P = 0.3$). On days 4 and 7 of infusion, the average urine sodium/creatinine ratio shows no significant difference between A-II treated and control rats (on day 4 of infusion, 3.2 ± 0.2 in A-II group, versus 2.7 ± 0.2 in controls, $P = 0.1$; on day 7 of infusion, 3.5 ± 0.3 in A-II group, versus 2.8 ± 0.2 in controls, $P = 0.07$).

3.3. A-II increases brush border membrane NHE3 activity in the rat renal cortex

BBMV were purified from the rat kidney cortex in A-II or saline-infused groups. Total BBMV pH-dependent Na^+ uptake was performed in the absence of HOE-694, reflecting

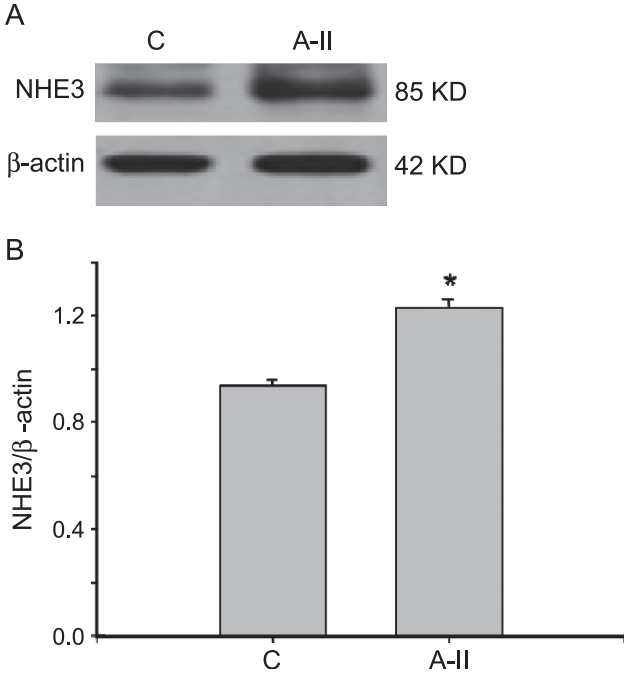

Fig. 4. Western blot analysis of NHE3 protein in rat renal cortical BBMV. Panel A shows one typical Western blot experiment. Western blots were reacted with NHE3-specific and β-actin-specific antiserum. The band at 85 kDa is NHE3 and the band at 42 kDa is β-actin. Panel B shows quantitative data from NHE3 Western blot experiments. Data are presented as a ratio of NHE3 to β-actin protein levels. Values are mean ± S.E.; number of replicates = 3; *$P < 0.05$.

Na^+ uptake mediated by HOE-694-sensitive and -insensitive NHEs. The total pH-dependent BBMV Na^+ uptake was not significantly altered by A-II treatment (data not shown), but HOE-694-insensitive Na^+ uptake was remarkably increased. As shown in Fig. 2, NHE3-mediated Na^+ uptake performed in the presence of 50 μM HOE-694 was increased ~ 88% with A-II treatment (2330.7 ± 267.3 pmol/mg protein/10 s in A-II groups; versus 1241.4 ± 131.6 in control groups; $n = 3$, $P = 0.005$).

3.4. Brush border membrane NHE2 protein abundance in the rat renal cortex was unchanged in response to A-II treatment

Western blot analyses with NHE2 antiserum showed specific recognition of an 85 kDa protein and A-II infusion didn't significantly change this immunoreactive NHE2 protein level, compared with controls (0.46 ± 0.01 densitometric units in A-II groups, versus 0.5 ± 0.06 in control groups; $n = 3$, $P = 0.55$) (Fig. 3).

3.5. A-II increases brush border membrane NHE3 protein abundance in the rat renal cortex

Western blot analyses with NHE3 antiserum showed specific recognition of an 85 kDa protein and A-II infusion increased the immunoreactive NHE3 protein band by 31%, compared with controls (1.23 ± 0.03 densitometric units in A-II groups, versus 0.94 ± 0.02 in controls; $n = 3$, $P = 0.02$) (Fig. 4).

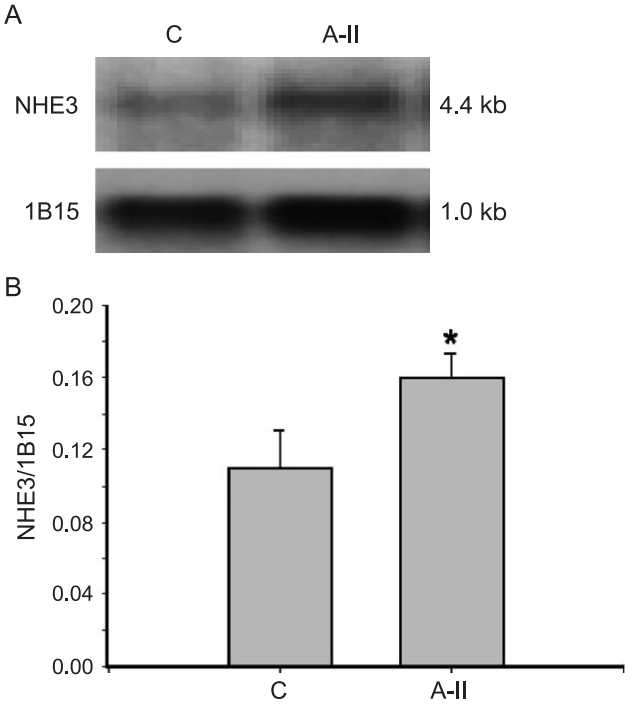

Fig. 5. Northern blot analysis of NHE3 mRNA expression in rat renal cortex. Panel A shows one typical Northern blot experiment. Northern blots were probed with NHE3-specific and 1B15-specific probes. The hybridization signal at 4.4 kilobase pairs (kb) represents rat NHE3, and hybridization signal at 1.0 kb represents 1B15. Panel B shows quantitative data from NHE3 Northern blot experiments. Data are presented as a ratio of NHE3 to 1B15 mRNA levels. Values are mean ± S.E.; number of replicates = 3. *$P < 0.05$.

3.6. A-II increases NHE3 mRNA abundance in the rat renal cortex

Northern blots were hybridized with NHE3-specific and 1B15-specific radiolabeled probes. As shown in Fig. 5, NHE3 mRNA abundance was increased by 42% after A-II administration, compared to controls (3.94 ± 0.13 densitometric units in A-II groups, versus 2.77 ± 0.21 in controls; $n = 3$, $P = 0.009$).

4. Discussion

The present study was designed to examine the effects of chronic A-II on Na^+/H^+ exchange isoform 2 and isoform 3. In the current study, we demonstrate that long-term A-II treatment increases apical NHE3-mediated Na^+ absorption, apical NHE3 protein abundance and NHE3 mRNA abundance. This increase in renal apical NHE3 activity might be partially due to the activation of NHE3 gene expression. We also demonstrate that the apical NHE2 transporter protein abundance is not affected by A-II treatment.

The present study utilized a previously established model of A-II-induced hypertension [27–31] to study the role of long-term A-II treatment on renal NHE2 and NHE3 regulation. In this study, we observed that A-II infusion significantly increases the systolic BP and plasma A-II levels, which was in agreement with other researchers [32]. The infusion dose of 500 ng min^{-1} kg^{-1} results in A-II plasma levels very similar to those found in the two-kidney one-clip Goldblatt model, which is widely used for renovascular hypertension studies [28].

In our study, A-II infusion significantly increased water intake above control levels, which is consistent with previous reports that circulating A-II can act on specific brain receptors to stimulate thirst [33]. We observed a large increase in the excretion of water, but no change in Na^+ excretion.

It has been shown that both NHE2 and NHE3 contribute to Na^+/H^+ exchange in the apical membrane of renal proximal tubule [34]. BBMV pH-dependent Na^+ uptake results suggest that HOE-694-sensitive transporters might have a counter-regulatory effect. Since NHE2 is a HOE-694-sensitive isoform ($IC_{50} = 5$ μM in PS120 cells) [23], we aimed to determine if the BBMV NHE2 protein is altered by A-II treatment. Western blot analysis of NHE2 showed that A-II treatment did not change BBMV NHE2 protein abundance; suggesting HOE-694-sensitive NHE isoforms other than NHE2 are involved. HOE-694 in 50 μM concentration is known to selectively inhibit NHE2 activity, therefore, Na^+ uptake with 50 μM HOE-694 represents

NHE3-mediated uptake [23,35]. Our results show that in the presence of 50 μM HOE-694 A-II increased pH-dependent Na^+ uptake by 88%.

To determine if the increase of HOE-694-insensitive Na^+ uptake is due to the increase in the apical NHE3 transporter, Western blotting was performed. Quantitation of bands from immunoblots with NHE3 antiserum showed that A-II infusion significantly increased NHE3 protein abundance in the brush border of the proximal tubules. This result is in agreement with a recent study [18].

In the our study, NHE3 functional activity increased by 88%, while BBMV NHE3 immunoblotting analysis showed a 31% increase in protein abundance. A possible explanation for this discrepancy may be that there can be post-translational modulation of NHE3 protein, such as phosphorylation/dephosphorylation regulation [36]. This modulated NHE3 protein cannot be detected by the NHE3 antibody used in our experiments.

To address if the NHE3 protein increase is due to an increase in NHE3 gene expression, Northern blot studies were performed. Our study shows a small but consistent and statistically significant increase in mRNA abundance. This change in mRNA expression parallels the change in immunoreactive protein expression. These results suggest that the increase in NHE3 protein after A-II treatment is likely due to increase in NHE3 gene transcription.

A recent study showed that the BBMV NHE3 protein abundance increased in response to A-II treatment through subcellular redistribution of NHE3 or differential protein–protein interaction [18]. The total NHE3 protein abundance was not altered. In our study, we did not examine total NHE3 protein abundance in the renal cortex. We studied the increase in BBMV NHE3 function that was due to increase in NHE3 mRNA abundance. This suggests that transcriptional regulation may be involved. The discrepancy between these two studies may be ascribed to the differences in rat species (Sprague–Dawley rats versus Munich–Wistar rats), A-II infusion dose (500 versus 200 ng min^{-1} kg^{-1}), feeding style (free access water/food versus restricted access), and other intangible factors.

Renal tubular effects of A-II are believed to be mediated by A-II type 1 (AT1)-receptor. Treatment with the AT1-receptor antagonist, candesartan, decreases the abundance of NHE3 in the brush border membrane from rat kidney proximal tubule [37]. This indicates that A-II regulation of NHE3 is likely to be mediated by the AT1 receptor. However, other researchers have shown that in A-II type 1a (AT_{1a}) receptor knockout mice maintained on a low-NaCl diet, NHE3 protein abundance shows no change as compared to wild-type [38]. This raises the possibility that the expression of NHE3 may be independent on AT_{1a} receptor. In our study, whether or not the effect of A-II on NHE3 activity was mediated by AT1 receptor was not assessed.

In summary, we have shown that long-term A-II infusion induces activity of apical NHE3 transporter in the proximal tubule through stimulating NHE3 mRNA and protein content. A-II treatment has no effect on renal NHE2 transporter protein abundance.

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