Angiotensin II-mediated biphasic regulation of proximal tubular Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 is impaired during oxidative stress

Anees Ahmad Banday and Mustafa F. Lokhandwala

Heart and Kidney Institute, College of Pharmacy, University of Houston, Houston, Texas

Submitted 1 March 2011; accepted in final form 14 May 2011

Banday AA, Lokhandwala MF. Angiotensin II-mediated biphasic regulation of proximal tubular Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 is impaired during oxidative stress. Am J Physiol Renal Physiol 301: F364–F370, 2011. First published May 18, 2011; doi:10.1152/ajprenal.00121.2011.—Angiotensin (ANG) II via AT1 receptors (AT1Rs) maintains sodium homeostasis by regulating renal sodium transporters including Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 (NHE3) in a biphasic manner. Low-ANG II concentration stimulates whereas high concentrations inhibit NHE3 activity. Oxidative stress has been shown to upregulate AT1R function that could modulate the ANG II-mediated NHE3 regulation. This study was designed to identify the signaling pathways responsible for ANG II-mediated biphasic regulation of proximal tubular NHE3 and the effect of oxidative stress on this phenomenon. Male Sprague-Dawley rats were chronically treated with a pro-oxidant L-buthionine sulfoximine (BSO) with and without an antioxidant tempol in tap water for 3 wk. BSO-treated rats exhibited oxidative stress and high blood pressure. At low concentration (1 pM) ANG II increased NHE3 activity in proximal tubules from all animals. However, in BSO-treated rats, the stimulation was more robust and was normalized by tempol treatment. ANG II (1 pM)-mediated NHE3 activation was abolished by AT1R blocker, intracellular Ca\textsuperscript{2+} chelator, and inhibitors of phospholipase C (PLC) and Ca\textsuperscript{2+}-dependent calmodulin (CaM) but it was insensitive to Gia and protein kinase C inhibitors or AT2R antagonist. A high concentration of ANG II (1 \muM) inhibited NHE3 activity in control and tempol-treated rats. However, in BSO-treated rats, ANG II (1 \muM) continued to induce NHE3 stimulation. Tempol restored the inhibitory effect of ANG II (1 \muM) in BSO-treated rats. The inhibitory effect of ANG II (1 \muM) involved AT1R-dependent, CGMP-dependent protein kinase (PKG) activation and was independent of AT2 receptor and nitric oxide signaling. We conclude that ANG II stimulates NHE3 via AT1R-PLC-CaM pathway and inhibits NHE3 by AT1R-PKG activation. Oxidative stress impaired ANG II-mediated NHE3 biphasic response in that stimulation was observed at both high- and low-ANG II concentration.

Address for reprint requests and other correspondence: A. A. Banday, Univ. of Houston, 4800 Calhoun Rd., 521 SR 2 Bldg., Houston, TX 77204 (e-mail: abanday@uh.edu).

The Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE), a plasma membrane-bound protein, is the major transporter of sodium across the luminal membrane of the proximal tubules (3, 21). Nine NHE isoforms have been identified in mammals and among them five are expressed in renal tissue (3, 21). The epithelial isoform NHE3 is responsible, in large measure, for sodium and hydrogen ion transport in the renal proximal convoluted tubule (3, 21). NHE3 is the prominent NHE isoform expressed in rat renal brush-border membranes and is mainly responsible for amiloride-sensitive Na\textsuperscript{+} transport across brush-border membrane (1). Studies using isolated membranes and reconstituted systems, conditions in which recycling or retrieval of NHE3 from the plasma membrane is unlikely, indicated that the membrane NHE3 activity can be regulated by phosphorylation/phosphorylation processes (1). Several hormones such as angiotensin (ANG) II, dopamine, and norepinephrine have been shown to regulate luminal NHE3 activity (8, 9, 25). ANG II, a major vasoconstrictor and strong mediator of sodium and volume regulation, activates NHE3 and stimulates sodium uptake in isolated proximal tubules (8, 25). The acute regulation of renal proximal tubular NHE3 activity by ANG II is biphasic; low concentrations stimulate the NHE3 activity, whereas high concentrations inhibit the transporter activity (15). However, the mechanisms of biphasic NHE3 regulation by ANG II remain unknown.

Since ANG II is a potent antinatriuretic hormone and the apical membrane Na\textsuperscript{+}/H\textsuperscript{+} antiport activity mediates ~100% of transcellular NaCl absorption in the proximal tubule, the stimulatory effect of ANG II on NHE3 has been extensively studied (8, 21). Microperfusion experiments in rat proximal tubules show that low circulating concentrations of ANG II, which enhance bicarbonate absorption, are associated with a decrease in tubular fluid cyclic AMP delivery. In these studies, luminal perfusion with dibutyryl cyclic AMP abolished the effect of ANG II (18). In addition, the effect of low circulating concentrations of ANG II is attenuated by pretreatment of rats with pertussis toxin (18). These experiments suggest that the effects observed may occur via protein kinase A inhibition. In separate experiments performed in opossum kidney cell line, the ANG II-induced increase in NHE3 activity is cyclic AMP independent and experiments in rat proximal tubules show that stimulation of NHE3 is abolished by protein kinase C (PKC) inhibitors (10, 19). Studies performed by Riquier-Brison and others (11, 25) showed that ANG II can also stimulate trafficking of NHE3 into the proximal tubule microvilli or increase its message levels. These data show that ANG II can activate renal NHE3 activity through more than one mechanism depending on the cell type and species. There is strong evidence that the regulation of NHE3 requires multiprotein signal complex (30). Interestingly, NHE3 COOH terminal possesses binding sites for regulatory proteins like Ca\textsuperscript{2+}-dependent calmodulin (CaM), which is readily stimulated by ANG II signaling (14). Since the role of CaM in ANG II-mediated NHE3 regulation is not clear, the present study will investigate the involvement of CaM in ANG II-induced NHE3 stimulation.

There are little mechanistic data available for the inhibitory effect of high doses of ANG II on NHE3. It is reported that high doses of ANG II increase cGMP level and inhibit Na-K-ATPase activity in renal proximal tubules (32). However, the mechanisms proximal to cGMP are still elusive. Also, the role of nitric oxide (NO) as a natriuretic factor is still not clear because millimolar concentrations of NO donors are required to raise cGMP levels and inhibit sodium transporters (6). However, natriuretic factors can increase proximal tubular...
cGMP levels via activation of particulate guanylyl cyclase and regulate sodium transporters (29). Therefore, this study is also aimed to identify the signaling molecules involved in ANG II-mediated NHE3 inhibition.

Most of the studies related to ANG II-mediated NHE3 regulation, at both low and high ANG II concentration, have been performed in proximal tubules from normotensive healthy animals or in epithelial cell cultures. There are reports that oxidative stress upregulates AT1 receptors that could modulate ANG II signaling (5, 17). The present study will examine the oxidative stress upregulates AT1 receptors that could modulate ANG II signaling (5, 17). The present study will examine the mechanisms by which ANG II exerts its biphasic effect on NHE3 activity. Additionally, we will also investigate the role of oxidative stress in modulating effects of ANG II on NHE3 regulation.

METHODS

Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN) and had free access to food and water. Animals were divided in four groups: 1) C (control) rats received tap water, 2) BSO, rats treated with 30 mM L-buthionine sulfoximine (Sigma, St. Louis, MO), 3) T, rats treated with 1 mM tempol (Sigma), and 4) BSO + T, rats received BSO plus tempol. Both BSO (a pro-oxidant) and tempol (an antioxidant) were given in tap water for 3 wk (28). These concentrations of BSO and tempol were chosen because both of the compounds are nontoxic when provided in drinking water (28). At the end of the treatment, blood pressure was measured as detailed previously (6).

Briefly, rats were anesthetized with Inactin (100 mg/kg ip) and the left carotid artery was catheterized with PE-50 tubing, connected to a Statham P23AC pressure transducer, and blood pressure and heart rate were recorded on a Grass polygraph (model 7D, Grass Instrument, Quincy, MA). Urinary 8-isoprostanate was measured by RIA kit (516351; Cayman, Ann Arbor, MI). Renal proximal tubular suspensions were prepared by using 20% Ficoll gradient in Krebs buffer as described previously (6). Protein was determined by using a bicinchoninic acid kit (Thermo Fisher, Houston, TX). Proximal tubular malondialdehyde was determined by the method of Uchiyama and Mihera (22). All the experimental procedures were approved by Institutional Animal Care and Use Committee.

Brush-border membrane vesicle NHE3 activity. The isolated proximal tubules were incubated with low concentration (1 mM) or high concentration (1 μM) of ANG II for 10 min at 37°C. Proximal tubules were also preincubated with the following pharmacological compounds before ANG II treatment: AT1 receptor antagonists candesartan (1 μM) and losartan (1 μM), AT2 receptor antagonist PD123319 (1 μM), Gastro inhibitor pertussis toxin (100 ng/ml), PLC inhibitors D609 (100 μM) and U73122 (10 μM), PKC inhibitors GF109203X (10 μM) and chelerythrine chloride (1 μM), intracellular calcium chelators BAPTA-AM (50 μM) and Quin2-AM (50 μM), CaM inhibitors W7 (100 μM) and ophioibol (10 μM), nitric oxide synthase (NOS) inhibitors l-NAME (1 mM) and l-NNA (100 μM), soluble guanylyl cyclase (sGC) inhibitors ODQ (40 μM) and NS2028 (10 μM), and cGMP-dependent protein kinase (PKG) inhibitors KT5823 (1 μM) and KT5822 (100 nM). Proximal tubules were homogenized and BBMVs were prepared as described previously (7). BBMVs were suspended in 150 mM KCl-25 mM MES buffer, pH 5.5 adjusted with KOH. These BBMVs were devoid of basolateral membrane sodium transporters Na+-K-ATPase and NHE1 as determined by immunoblotting (data not shown). The specific activity of apical membrane enzymes alkaline phosphatases and γ-glutamyltransferase was 15- to 20-fold higher in BBMV compared with cell homogenate (data not shown) (7). To determine the vesicle volume/size, 32Na+ and 32P uptake was determined at 1- to 2-h incubation, assumed to present equilibrium values. We did not observe any difference in BBMV 22Na+ and 32P uptake among various animal groups or between vehicle- and drug-treated proximal tubules, suggesting that there was no variation in BBMV size between preparations (data not shown).

Activities of BBMV marker enzymes and 32P transport were determined as detailed previously (7). BBMV NHE3 activity was determined by measurement of 100 μM 5-(N-methyl-N-isobutyl)-amiloride-sensitive 22Na+ uptake. 32Na+ uptake was insensitive to 5-(N-ethyl-N-isopropyl)-amiloride (1 μM), indicating that uptake was due to NHE3 and is not contributed by NHE1 which is 100-fold more sensitive to 5-(N-ethyl-N-isopropyl)-amiloride than NHE3 (1). The uptake of 22Na+ into BBMV was measured at 24°C by mixing 15 μl of the membrane vesicle suspension (~200 μg protein) and 25 μl of uptake medium [142 mM KCl, 14.7 mM KOH, 10 mM MES, 9 mM HEPES, and 1 mM NaCl (containing 0.2 μCi of 22Na+), pH 7.4, pH in 5.5, pHout 7.4, [Na+]in = 0 mM, and [Na+]out = 1 mM]. The reaction was stopped by Millipore rapid filtration technique with 0.45-μm nitrocellulose filters. Na+ uptake in proximal tubular BBMVs is predominantly from NHE3 activity; however, proximal tubular apical membranes also express NHE8.

The chemicals were obtained from the following sources: candesartan was a kind gift from AstraZeneca (Wilmington, DE); PD123319 was from R&D Systems (Minneapolis, MN); BAPTA-AM, pertussis toxin, GF109203X, W7, l-NAME, and l-NNA were from Calbiochem (San Diego, CA); D609 and U73122 were purchased from Biomol Research Labs (Plymouth Meeting, PA); ODQ, NS2028, and KT5823 were from Cayman; ANG II, D609, 5-(N-methyl-N-isobutyl)-amiloride, 5-(N-ethyl-

Fig. 1. Oxidative stress markers and mean blood pressure in control (C), L-buthionine sulfoximine (BSO), tempol (T), and BSO + T (BSO+T)-treated rats. A: urinary 8-isoprostanate excretion normalized with urinary creatinine. B: protein oxidation measured as nitrotyrosine in renal proximal tubular homogenates. C: mean blood pressure. *P < 0.05 vs. C and #P < 0.05 vs. BSO, using 1-way ANOVA followed by Newman-Keuls post hoc test.
N-isopropyl)-amiloride, Quin2-AM, and other chemicals were obtained from Sigma.

**Immunoblotting.** Proximal tubules were homogenized in buffer containing 150 mM NaCl, 50 mM Tris HCl, pH 7.4, 1 mM EDTA, 1% NP-40, 1 mM NaF, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Complete, Roche Diagnostics, Indianapolis, IN) mixed with Laemmli buffer (16), and Western blotting was performed as detailed previously (6). Briefly, the samples were subjected to SDS-PAGE and transferred to PVDF membrane that was incubated with NHE3, NHE1, megalin, Na⁺-K⁺-ATPase (α1-subunit), nitrotyrosine, or GAPDH antibodies at 4°C overnight. Antibodies for NHE3 (catalog no. MAB3136 and AB3085), NHE1 (catalog no. MAB3140 and AB3081), GAPDH (MAB374), nitrotyrosine (catalog no. 05–233), and Na-K-ATPase (catalog no. 05–369) were obtained from Millipore (Billerica, MA). The bands were visualized by ECL reagent (Millipore) and quantitated by Kodak Imaging System (Rochester, NY).

**PKG activity assay.** Proximal tubules were incubated with 1 μM ANG II and homogenized. The lysates were centrifuged at 8,000 g at 4°C for 30 s, and the supernatant fractions were used for kinase assay. PKG activity was determined as described by Fiscus and Murad (13). Briefly, the amount of 32P transferred from [γ-32P]ATP to PKG substrate BPDeite (Biomol Research Labs) was measured by adding 7 μl of supernatant fraction of tissue homogenates to 35 μl of reaction mixture containing 15 mM K2PO4, pH 7.0, 7 mM magnesium acetate, 200 μM BPDeite, 20 mM NaF, 0.25 mM isobutylmethylxanthine, 30 μM ATP with [γ-32P]ATP, and 0.3 μM KT5720 (a highly specific inhibitor of cAMP kinase). The reaction was stopped by transferring 35 μl of final reaction mixture to Whatman No. 3MM chromatography paper squares and radioactivity was determined by liquid scintillation counter (Beckman, Brea, CA).

**Statistical analysis.** Differences between means were evaluated by using ANOVA with Newman-Keuls multiple test, as appropriate. *P < 0.05 was considered statistically significant. We used two to three rats (for every experimental group, i.e., control, BSO, T, and BSO+T) for proximal tubular preparation for various pharmacological treatments and four to five tubular preparations were used for this study (n = 4–5 and number of animals used for each group ∼10–15). Biochemical analysis was performed in triplicate in every tubular preparation.

**RESULTS**

Treatment of animals with BSO or tempol for 3 wk had no effect on body weight or food/water intake (data not shown). BSO-treated rats exhibited oxidative stress as evidenced by increased urinary 8-isoprostane excretion and renal proximal tubular protein nitrosylation (Fig. 1, A and B). These rats also exhibited higher mean blood pressure compared with control rats (Fig. 1C). Tempol supplementation to BSO-treated rats mitigated oxidative stress and normalized blood pressure (Fig. 1, A–C). Tempol, per se, had no effect on body weight or levels of oxidative stress markers (Fig. 1, A–C).

**ANG II-mediated NHE3 stimulation.** Renal proximal tubules were incubated with low concentration of ANG II (1 pM) for 10 min at 37°C and BBMVs were prepared as detailed previously (7). Incubation of renal proximal tubules with ANG II (1 pM) increased 22Na⁺ uptake in BBMVs from all experimental groups, suggesting NHE3 stimulation (Fig. 2A). The NHE3 stimulation was significantly higher in BSO-treated compared with control rats (Fig. 2A). Tempol normalized the ANG II-mediated overstimulation of NHE3 in BSO-treated rats (Fig. 2A). Response to ANG II in tempol-treated rats was comparable with control rats (Fig. 2A). Preincubation of BBMVs with NHE3 inhibitor 5-(N-methyl-N-isobutyl)-amiloride (100 μM) blocked 22Na⁺ uptake (Fig. 2A). Incubation of proximal tubules with candesartan or losartan (AT1 receptor antagonists) abolished ANG II-mediated NHE3 activation while PD123319 (AT2 receptor antagonist) had no effect (Fig. 2B). The basal NHE3 protein expression was similar in all experimental groups (Fig. 2C). Incubation of proximal tubules with ANG II receptor blockers did not change the basal 22Na⁺ uptake (vehicle: 3.2 ± 0.2, candesartan: 3.0 ± 0.3, losartan: 3.3 ± 0.3, PD123319: 3.2 ± 0.2; in nmol·mg protein⁻¹·min⁻¹).

**AT1 receptor signaling involved in NHE3 stimulation.** To identify which G protein is involved in ANG II (1 pM)-mediated NHE3 activation, proximal tubules were incubated with Gqi inhibitor pertussis toxin or PLC inhibitors D609 and U73122. As shown in Fig. 3A, pertussis toxin had no effect on ANG II-mediated NHE3 stimulation in proximal tubules from any experimental group. However, PLC inhibitors D609 and

---

**Fig. 1.** Effect of lower (1 pM) angiotensin (ANG) II concentration on Na⁺/H⁺ exchanger 3 (NHE3) activity in renal proximal tubules from C, BSO-, T-, and BSO+T-treated rats. A: proximal tubules were incubated with ANG II and NHE3 activity (22Na⁺ uptake) was measured in brush-border membrane vesicles in the presence and absence of NHE3 inhibitor 5-(N-methyl-N-isobutyl)-amiloride (MIA). B: proximal tubules were incubated with AT1 receptor antagonists candesartan and losartan or AT2 receptor antagonist PD123319 before ANG II exposure. C: NHE3 expression in brush-border membrane vesicles. *P < 0.05 vs. vehicle and #P < 0.05 vs. ANG II from BSO-treated rats, using 1-way ANOVA followed by Newman-Keuls post hoc test.
U73122 abolished ANG II effect, suggesting that stimulation of NHE3 is mediated via Gq activation (Fig. 3A). Since PLC can activate PKC and increase cellular Ca$^{2+}$ levels, we used PKC inhibitors (GF109203X and chelerythrine chloride) and Ca$^{2+}$ chelators (BAPTA-AM and Quin2-AM) to study their involvement in NHE3 stimulation. Incubation of proximal tubules with GF109203X and chelerythrine chloride before ANG II exposure had no effect on NHE3 stimulation (Fig. 3B). However, Ca$^{2+}$ chelators blocked the ANG II-mediated NHE3 activation (Fig. 3B). Furthermore, CaM inhibitors W7 and ophiobolin also abolished hormonal stimulation of NHE3 (Fig. 3B). These pharmacological compounds had no effect on basal NHE3 activity ($^{22}$Na$^{+}$ uptake: vehicle: $3.3 \pm 0.3$, pertussis toxin: $3.2 \pm 0.4$, D609: $3.1 \pm 0.4$, U73122: $3.2 \pm 0.2$, GF109203X: $3.0 \pm 0.4$, chelerythrine chloride: $3.2 \pm 0.3$, BAPTA-AM: $3.1 \pm 0.4$, Quin2-AM: $3.3 \pm 0.3$, W7: $3.1 \pm 0.3$, and ophiobolin: $3.2 \pm 0.4$; in nmol-mg protein$^{-1}$.min$^{-1}$).

**AT1 receptor signaling involved in NHE3 inhibition.** Incubation of proximal tubules with higher ANG II concentration ($1 \mu$M) inhibited the NHE3 activity in control rats (Fig. 4A). Similar response to micromolar ANG II concentration was seen in tempol-treated rats (Fig. 4A). However, in BSO-treated rats, ANG II stimulated NHE3 at high concentration (Fig. 4A). Tempol supplementation to BSO-treated rats restored the inhibitory response to micromolar ANG II concentration (Fig. 4A). To identify the signaling molecules responsible for inhibitory effect of ANG II, proximal tubules were preincubated with candesartan or PD123319. While candesartan abolished both the inhibitory effect of ANG II in control, tempol-, and BSO plus tempol-treated rats and stimulatory effect in BSO-treated rats, PD123319 had no effect (Fig. 4A). To determine whether NO pathway is involved in the inhibitory response to ANG II, proximal tubules were incubated with NOS inhibitors L-NAME and l-NNa or sGC inhibitors ODQ and NS2028. As shown in Fig. 4B, inhibitors of NOS and sGC failed to block the ANG II-mediated NHE3 inhibition in control, tempol-, and BSO plus tempol-treated rats or stimulation in BSO-treated rats (Fig. 4B). NOS or sGC inhibitors had no effect on basal $^{22}$Na$^{+}$ uptake (vehicle: $3.1 \pm 0.3$, l-NAME: $3.2 \pm 0.3$, l-NNa: $3.3 \pm 0.1$, ODQ: $3.1 \pm 0.3$, NS2028: $3.2 \pm 0.4$; in nmol-mg protein$^{-1}$.min$^{-1}$).

**ANG II-mediated NHE3 inhibition involves PKG.** Incubation of proximal tubules with PKG inhibitors KT5922 or KT5823 abolished ANG II ($1 \mu$M)-induced NHE3 inhibition in control rats (Fig. 5). Similar effects were seen in tempol- and BSO plus tempol-treated rats (Fig. 5). In these rats, PKG inhibition restored the stimulatory response to high-ANG II concentration (Fig. 5A). PKG inhibitors had no effect in BSO-treated rats (Fig. 5). Also, these inhibitors did not change the basal NHE3 activity ($^{22}$Na$^{+}$ uptake: vehicle: $3.2 \pm 0.2$, KT5922: $3.0 \pm 0.2$, KT5823: $3.1 \pm 0.3$).

**PKG activation.** ANG II-induced PKG activation was measured in proximal tubular homogenates as histone phosphorylation. Incubation of proximal tubules with $1 \mu$M ANG II significantly increased PKG activity in control and tempol-treated rats (Fig. 6). The activation of PKG was blocked by AT1 receptor antagonist candesartan and PKG inhibitor KT5823 (Fig. 6). AT2 receptor blocker PD123319 had no effect (Fig. 6). However, ANG II ($1 \mu$M) failed to activate PKG in proximal tubules from BSO-treated rats (Fig. 6). Tempol supplementation of BSO-treated rats restored ANG II-induced PKG activation (Fig. 6). Basal PKG activity was similar in all experimental groups (Fig. 6).
DISCUSSION

Our results demonstrate that in normotensive animals ANG II regulates renal proximal tubular apical NHE3 in biphasic manner. Stimulation of NHE3 at lower ANG II concentration involves AT1 receptor-mediated PLC activation, intracellular Ca\(^{2+}\)/H\(_{11001}\) mobilization, and CaM stimulation. On the other hand, the inhibition of renal apical NHE3 in response to high-ANG II concentration is mediated by AT1 receptor-dependent PKG activation. Furthermore, in BSO-treated rats exhibiting oxidative stress and high blood pressure, the biphasic regulation of NHE3 in response to ANG II is lost in that stimulation is observed at high concentration. Also, the ANG II-induced stimulation of NHE3 is significantly higher in BSO-treated rats. In these animals, ANG II failed to activate PKG. Tempol supplementation of BSO-treated rats reduced oxidative stress, normalized ANG II signaling and biphasic regulation of NHE3, and reduced blood pressure.

ANG II can directly increase renal tubular sodium reabsorption by regulating proximal tubular sodium transporters including NHE3 (21, 25). NHE3 is expressed exclusively on apical side of proximal tubules and plays an important role in Na\(^{+}\) reabsorption. NHE3 knockout mice have reduced proximal tubular Na\(^{+}\)/H\(_{11001}\) reabsorption that leads to Na\(^{+}\) wasting (20). It is reported that ANG II stimulates NHE3 at physiological concentrations and this activation is sensitive to AT1 receptor antagonists (5, 25). In the present study, we also found that incubation of proximal tubules with low-ANG II concentration activated NHE3. In BSO-treated animals, which exhibited oxidative stress and high blood pressure, the activation of NHE3 in response to ANG II was more robust than control rats. It is reported that oxidative stress can upregulate AT1 receptor signaling in various hypertensive models (5, 24). Also, antioxidant supplementation has been shown to normal-
ize AT1 receptor signaling in animals exhibiting oxidative stress (5). The present study also shows that tempol supplementation to BSO-treated rats reduced oxidative stress, normalized ANG II-mediated NHE3 stimulation, and decreased blood pressure. The hormonal activation of NHE3 was blocked by candesartan and not by PD123319, suggesting that NHE3 activation is AT1 receptor specific. Therefore, during oxidative stress, the upregulation of AT1 receptors could lead to higher NHE3 stimulation at physiological ANG II concentration, which in turn could increase sodium absorption and may contribute to high blood pressure. There are reports of enhanced proximal tubular sodium reabsorption, lower Na\(^+\) excretion, and greater NHE3 activity in young spontaneously hypertensive rats (SHR) (2, 27). Although increased proximal tubular fluid and sodium reabsorption could contribute to sodium retention and development of hypertension in SHR, the overactive sodium transport is not seen in older SHR (4). Also, there are reports that either failed to show difference in sodium reabsorption between adult SHR and Wistar-Kyoto rats or observed reduced proximal tubular fluid reabsorption and NHE3 activity in SHR (4, 23, 31). The exact mechanisms for these discrepancies are not clear; however, it is possible that age or strain difference could play an important role in renal sodium regulation.

The mechanisms involving stimulation of NHE3 by ANG II are conflicting. While some studies show that ANG II-mediated NHE3 activation in proximal tubules is PKC dependent, others showed the involvement of PKA and IP3 kinase (12, 19, 30). The stimulation of NHE3 has also been shown to be both dependent and independent of changes in membrane expression of this transporter (5, 11). Since AT1 receptors are coupled to both Gi and Gq proteins and both of these G proteins have been implicated in NHE3 stimulation, we first used Gia and PLC (activated upon AT1 receptor-Gq coupling) inhibitors (5, 15). We found that PLC inhibition abolished ANG II-induced NHE3 stimulation, whereas pertussis toxin had no effect, suggesting that AT1 receptor-Gq-PLC pathway is involved in NHE3 regulation. Since this pathway can activate PKC and raise intracellular Ca\(^{2+}\) levels, we used PKC inhibitors and Ca\(^{2+}\) chelators to identify the molecules involved in NHE3 activation proximal to PLC. Our data show that PKC inhibitors failed to block ANG II signaling while stimulation of NHE3 was sensitive to intracellular Ca\(^{2+}\) availability. The increase in cellular Ca\(^{2+}\) can activate a plethora of pathways including stimulation of CaM (14). Interestingly, we found that inhibition of CaM abolished ANG II-induced NHE3 activation. Although we did not study the mechanism for CaM-NHE3 interaction, a recent study shows that Ca\(^{2+}\) can activate NHE3 by increasing its interaction with IP3 receptor binding protein and this interaction is CaM dependent (14). Therefore, these data suggest that at low concentration, ANG II activated NHE3 via AT1 receptor-Gq-PLC-CaM pathway and this pathway is involved in both normotensive and hypertensive animals.

At low concentration, ANG II stimulates sodium transporters while high-ANG II concentrations inhibit these transporters (15, 32). The present study also shows that in normotensive animals, high concentration of ANG II significantly inhibited NHE3 activity. However, in BSO-treated rats, ANG II not only failed to inhibit the NHE3 at higher concentration but continued to show robust stimulation of this transporter. The inhibitory response was restored in tempol-supplemented, BSO-treated rats. These data suggest that oxidative stress impairs the inhibitory hormonal effect on NHE3 that may decrease sodium excretion and could contribute to hypertension. Antioxidant treatment restored the NHE3 biphasic response and decreased blood pressure.

There are reports that ANG II can inhibit Na-K-ATPase activity via NO signaling (32). To determine whether NO pathway is involved in ANG II-induced NHE3 inhibition, renal proximal tubules were incubated with inhibitors of NOS and sGC. Both NOS and sGC inhibitors failed to prevent the NHE3 inhibition in control or tempol-treated rats and stimulation in BSO-treated rats at high-ANG II concentration. Surprisingly, in control and tempol-treated rats, PKG inhibitors not only abolished the ANG II-induced NHE3 inhibition but also restored the ANG II-mediated NHE3 stimulation. Further studies showed that high-ANG II concentration activated PKG in control and tempol-treated rats but not in BSO-treated rats. Tempol supplementation restored the ANG II-induced NHE3 inhibition and PKG activation in BSO-treated rats. These data show that at high concentration ANG II inhibits NHE3 via PKG activation. It has been shown that natriuretic peptides inhibit renal Na\(^+\)-K\(^+\)-ATPase activity via natriuretic peptide receptor-cGMP-PKG pathways (29). Since oxidative stress can reduce the cGMP-induced PKG stimulation (26), the failure of ANG II to activate PKG could explain the loss of biphasic response in BSO-treated rats. Taken together, the involvement of PKG, independent of NO-cGMP pathway, in ANG II-mediated NHE3 inhibition identifies a novel pathway for sodium regulation in proximal tubules.

In conclusion, our data show that ANG II regulates NHE3 activity in a biphasic manner in normotensive animals. At low concentration (pM), ANG II stimulated NHE3 via AT1 receptor-PLC-CaM pathway. At high-ANG II concentration (μM), ANG II inhibits NHE3 activity via AT1 receptor-PKG pathway. During oxidative stress, ANG II, at lower concentration, causes higher NHE3 stimulation. Also, the inhibitory response at high-ANG II concentration is lost and instead stimulation is observed. This phenomenon could increase sodium reabsorption and may contribute to hypertension. Antioxidant supplementation reduces oxidative stress, normalizes AT1 receptor signaling, and mitigates hypertension.

GRANTS

This study was supported by Scientist Development Grant 0835428N from the American Heart Association-National Center to A. A. Banday.

DISCLOSURES

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

REFERENCES

BIPHASIC REGULATION OF RENAL NHE3 BY ANGIOTENSIN II


