Defective nitric oxide production impairs angiotensin II-induced Na-K-ATPase regulation in spontaneously hypertensive rats

Apurva A. Javkhedkar, Mustafa F. Lokhandwala, and Anees Ahmed Banday

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

Submitted 17 May 2011; accepted in final form 2 September 2011

Javkhedkar AA, Lokhandwala MF, Banday AA. Defective nitric oxide production impairs angiotensin II-induced Na-K-ATPase regulation in spontaneously hypertensive rats. Am J Physiol Renal Physiol 302: F47–F51, 2012. First published September 7, 2011; doi:10.1152/ajprenal.00270.2011.—Angiotensin (ANG) II via ANG II type 1 receptors (AT1R) activates renal sodium transporters including Na-K-ATPase and regulates sodium homeostasis and blood pressure. It is reported that at a high concentration, ANG II either inhibits or fails to stimulate Na-K-ATPase. However, the mechanisms for these phenomena are not clear. Here, we identified the signaling molecules involved in regulation of renal proximal tubular Na-K-ATPase at high ANG II concentrations. Proximal tubules from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were incubated with low concentrations of ANG II (pM), which activated Na-K-ATPase in both the groups; however, the stimulation was more robust in SHR. A high concentration of ANG II (µM) failed to stimulate Na-K-ATPase in WKY rats. However, in SHR ANG II (µM) continued to stimulate Na-K-ATPase, which was sensitive to the AT1R antagonist candesartan. In the presence of 1,2-arginine methyl ester (L-NAME), a nitric oxide (NO) synthase (NOS) inhibitor, ANG II (µM) caused stimulation of Na-K-ATPase in proximal tubules of WKY rats while having no further stimulatory effect in SHR. ANG II (µM), via AT1R, increased proximal tubular NO levels in WKY rats but not in SHR. In SHR, NOS was uncoupled as incubation of proximal tubules with ANG II and L-arginine, a NOS substrate, caused superoxide generation only in SHR and not in WKY rats. The superoxide production in SHR was sensitive to L-NAME. There was exaggerated proximal tubular AT1R-G protein coupling and NADPH oxidase activation in response to ANG II (µM) in proximal tubules of SHR compared with WKY rats. In SHR, inhibition of NADPH oxidase restored NOS coupling and ANG II-induced NO accumulation. In conclusion, at a high concentration ANG II (µM) exerts its effect on Na-K-ATPase is not completely understood. It is reported that high concentrations of ANG II via AT1R can activate nitric oxide (NO)-cGMP signaling in rat proximal tubules (12, 24). Studies show that defective NO signaling can influence ANG II- mediated Na-K-ATPase regulation (1). Interestingly, ANG II is known to activate NADPH oxidase and cause superoxide production. Superoxides can reduce NO bioavailability by either formation of peroxynitrite or by uncoupling NO synthase (NOS). We have reported that defective NO signaling can affect Na-K-ATPase regulation at high concentrations of ANG II and thus can contribute to hypertension in Sprague-Dawley rats treated with prooxidant 1-buthionine sulfoximine (1). Therefore, the present study was designed to determine the involvement of the NO system in AT1R-mediated Na-K-ATPase regulation at high concentrations of ANG II in spontaneously hypertensive rats (SHR), a genetic model of essential hypertension, and their normotensive control Wistar-Kyoto (WKY) rats.

METHODS

Animals. Male 12- to 14 wk-old SHR and WKY rats were purchased from Charles River Laboratories (Wilmington, MA). The rats were placed in plastic cages with free access to tap water and standard rat chow (Purina Mills, St. Louis, MO). All experiments were performed according to University of Houston guidelines, and protocols were approved by the Institutional Animal Care and Use Committee. Animal surgery. Implantable radio transmitters (TA11PA-C40; Data Sciences, St. Paul, MN) were used to measure conscious blood pressure in individual animals. Anesthesia was induced by 5% isoflurane and maintained by 2.5–3% of isoflurane with a face mask throughout the surgery. After a midline abdominal incision was made, the abdominal aorta was exposed by using sterile cotton swabs. The catheter of the telemetric device was inserted into the abdominal aorta and guided upstream. To secure the catheter to the aorta, tissue adhesive (Vetbond, 3M Animal Care Products, St Paul, MN) was used. The body of the telemetric device was placed in the abdominal cavity and sutured to the abdominal musculature. The abdominal musculature and skin were closed individually. The surgery was performed under sterile conditions on a heating pad (37°C). Analgesia was achieved by the administration of buprenorphine (0.05 mg/kg sq).
every 12 h for 2 days. Animals were allowed 1 wk for recovery before blood pressure measurements were recorded.

Preparation of renal proximal tubules. Proximal tubules were prepared as described previously (6). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and, after a midline incision, the aorta was cannulated below the kidneys. In situ digestion was accomplished by perfusing an enzyme solution of 230 U/ml collagenase and 250 U/ml hyaluronidase (Sigma, St. Louis, MO). Enrichment of proximal tubules was carried out using a 25% Ficoll density gradient in Krebs-Henseleit buffer B (KHB-B) without calcium [KHB-B (in mM): 118 NaCl, 27.2 NaHCO3, 4 KCl, 0.12 MgCl2, 1 KH2PO4, 5 glucose, and 10 HEPES, pH 7.4]. The band at the Ficoll-KHB-B buffer interface was collected and washed with KHB (5) by centrifugation at 250 g for 5 min. Proximal tubular viability was checked by using a trypan blue exclusion test. Protein was determined by using a bicinchoninic acid kit (Pierce Chemicals).

Na,K-ATPase activity. Na-K-ATPase activity was determined as described by Quigley and Gotterer (18). Briefly, renal proximal tubules (1 mg protein) were incubated without (basal) and with ANG II (10^-6 or 10^-8 M) at 37°C for 15 min in a water bath. After incubation, the proximal tubules were lysed by snap freezing in liquid nitrogen. The lysed tubules were used to assay ouabain (4 mM)-sensitive Na-K-ATPase activity, which was determined by phosphate hydrolysis of ATP (4 mM). The inorganic phosphate released was determined colorimetrically as described by Taussky and Shorr (21).

[^35]S[GTP-γS binding assay. The assay was performed as described earlier (11). Briefly, the reaction mixture containing 100 µl of 0.6 nM[^35]S[GTPγS, ANG II (10^-6 M), and 5 µg of proximal tubular membrane protein was incubated at 30°C for 60 min. Nonspecific[^35]S[GTP-γS binding was determined in the presence of 100 µM unlabeled GTP-γS. The reaction was stopped by rapid filtration on GF/C filters under vacuum, and radioactivity was determined by a liquid scintillation counter (Beckman Coulter, Brea CA).

Immuno blotting. Proximal tubular homogenate was used to immunoblot AT1R and glyceraldehyde 3-phosphate dehydrogenase. Proteins were solubilized in Laemmli buffer (13), resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (Pierce Chemicals) and incubated with antibodies directed against AT1R (Santa Cruz Biotechnology, Santa Cruz, CA) and glyceraldehyde 3-phosphate dehydrogenase (Calbiochem, La Jolla, CA) in 0.1% Tris-buffered saline followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The signal was detected with enhanced chemiluminescence substrate (Santa Cruz Biotechnology) and quantified by Kodak Imaging System (Rochester, NY).

Determination of NO level. Renal proximal tubules were incubated without (basal) or with ANG II (10^-6 M) in the presence or absence of AT1R blocker candesartan (1 µM), and tissues were snap frozen in liquid nitrogen followed by homogenization. The levels of NO were determined colorimetrically using a nitrate/nitrite assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s protocol.

**Superoxide detection.** Fluorescence spectrometric assay of superoxide production was performed as detailed by Satoh et al. (20). Briefly, proximal tubules were incubated with and without ANG II (10^-6 M) for 15 min. Oxidation of dihydroethidium to ethidium in the presence of NADH or L-arginine was used as a measure of superoxide production. Conversion of dihydroethidium to ethidium in the absence of ANG II was considered as basal superoxide production.

**Statistics analysis.** Differences between the means were evaluated using an unpaired Student’s t-test or one-way ANOVA with a Newman-Keuls multiple test, as appropriate. *P* < 0.05 was considered statistically significant.

**RESULTS**

Both, SHR and WKY rats had similar food (WKY 19.4 ± 2.0, SHR 18.6 ± 1.0, g/day) and water (WKY 40.0 ± 3.5, SHR 38.5 ± 2.6 ml/day) intake and body weights (WKY 309.3 ± 5.0, SHR 297.5 ± 4.0 g). Compared with WKY rats, SHR had significantly higher mean arterial pressure (WKY 98.7 ± 1.3, SHR 134.7 ± 1.5 mmHg).

**Effect of ANG II on proximal tubular Na-K-ATPase activity.** Incubation of proximal tubules with a low concentration of ANG II (10^-10 M) caused stimulation of Na-K-ATPase in both SHR and WKY rats (Fig. 1A). However, ANG II-induced stimulation of Na-K-ATPase was significantly higher in SHR compared with WKY rats (Fig. 1A). A higher concentration of ANG II (10^-6 M) failed to activate Na-K-ATPase in WKY rats; yet, at this concentration, ANG II continued to stimulate Na-K-ATPase in SHR (Fig. 1A). The basal Na-K-ATPase activity was similar in SHR and WKY rats (Fig. 1B). As shown in Fig. 1C, stimulation of Na-K-ATPase by ANG II (10^-6 M) in SHR was sensitive to the AT1R antagonist candesartan (1 µM).

**Role of NO in ANG II-mediated Na-K-ATPase regulation.** To investigate whether NO has a role in ANG II (10^-6 M)-mediated Na-K-ATPase regulation, we pretreated proximal tubules of SHR and WKY rats with NOS inhibitor N^6^-nitro-L-arginine methyl ester (L-NAME). In WKY rats, L-NAME (1 mM) treatment resulted in significant stimulation of Na-K-ATPase by ANG II (Fig. 2A). However, L-NAME treatment did not change ANG II-induced Na-K-ATPase stimulation in SHR (Fig. 2A).

Incubation of proximal tubules with ANG II (10^-6 M) caused a significant increase in NO production in WKY rats,
but ANG II did not significantly increase NO production in SHR (Fig. 2B). ANG II-induced NO production in WKY rats was sensitive to the AT1R antagonist candesartan (1 μM) (Fig. 2B). The basal levels of NO were similar in both SHR and WKY rats (Fig. 2B). However, ANG II (10⁻⁶ M) increased membrane [³⁵S]GTPγS binding was significantly higher in SHR compared with WKY rats (Fig. 4B). Basal [³⁵S]GTPγS binding was similar in SHR and WKY rats (Fig. 4C).

DISCUSSION

The results of our study show that in WKY rats ANG II regulates proximal tubular Na-K-ATPase in a biphasic manner in that at a low concentration (pM) it activates Na-K-ATPase while at a high concentration (μM) there is loss of stimulation. In these rats, ANG II (μM) increased proximal tubular NO levels and l-NAME restored the stimulation of Na-K-ATPase at a higher ANG II concentration, indicating the role of NO in loss of stimulation. On the other hand, in SHR ANG II caused exaggerated stimulation of Na-K-ATPase at a lower concentration and continued the stimulation at a higher concentration as well. In these rats, NOS was uncoupled as ANG II (μM) failed to increase tubular NO levels and coincubation of tubules from SHR and WKY rats. Values are means ± SE from 6 – 8 rats. *P < 0.05 vs. basal. #P < 0.05 vs. WKY for ANG II (10⁻⁶ M). $P < 0.05 vs. WKY for ANG II (10⁻⁶ M) using 1-way ANOVA followed by a Newman-Keuls multiple test.

Renal proximal tubular ATIR protein expression and G protein coupling. Western blot analysis showed similar AT1R protein expression in proximal tubules of SHR and WKY rats (Fig. 4A). Incubation of proximal tubules with ANG II (10⁻⁶ M) increased membrane [³⁵S]GTPγS binding in both SHR and WKY rats (Fig. 4B). However, ANG II-induced membrane [³⁵S]GTPγS binding was significantly higher in SHR compared with WKY rats (Fig. 4B). Basal [³⁵S]GTPγS binding was similar in SHR and WKY rats (Fig. 4C).
bules with ANG II (μM) and l-arginine caused superoxide generation. Also, in these animals there was higher proximal tubular AT1R-G protein coupling and NAD(P)H oxidase activation in response to ANG II (μM). NAD(P)H inhibition restored NOS coupling as incubation of proximal tubules with ANG II (μM) restored NO generation, which also abolished ANG II (μM)-induced Na-K-ATPase activation in SHR. Taken together, our data suggest that in WKY rats the loss of ANG II (μM)-induced Na-K-ATPase stimulation is mediated via a NO pathway. However, in SHR higher NAD(P)H oxidase activation caused NOS uncoupling and impaired the NO system, which leads to continued ANG II (μM)-induced stimulation.

Renal proximal tubular Na-K-ATPase, expressed on basolateral membranes, plays an important role in sodium transport, and studies show that perturbation in Na-K-ATPase activity can lead to sodium imbalance and thereby contribute to hypertension. The regulation of Na-K-ATPase in response to ANG II is such that picomolar-nanomolar concentrations of ANG II either inhibits or fails to stimulate Na-K-ATPase (3, 9). In agreement with these studies, we observed a loss of Na-K-ATPase stimulation at high concentrations of ANG II in WKY rats. It is worth noting that at low concentrations of ANG II we observed stimulation of Na-K-ATPase in both SHR and WKY rats, suggesting that AT1R are functional in proximal tubular preparations. Interestingly, in SHR we observed stimulation of Na-K-ATPase at high concentrations of ANG II, and this stimulation was sensitive to an AT1R antagonist, suggesting the involvement of AT1R. Navar et al. (16, 17) have reported that the proximal tubular concentration of ANG II is much higher than the plasma concentration because there are two sources of ANG II in proximal tubules, local synthesis and glomerular filtration, and there is a further increase in intrarenal ANG II levels during pathological conditions such as hypertension. Thus increase in intrarenal ANG II levels may contribute to sodium retention and hypertension if sodium transporter stimulation is maintained at high concentrations. Therefore, it is possible that stimulation of Na-K-ATPase at high concentrations of ANG II may cause an increase in sodium reabsorption and contribute to hypertension in SHR.

Stimulation of Na-K-ATPase in response to low concentrations of ANG II is shown to be mediated by various protein kinases, including MAP kinase and protein kinase C (1–3). However, the mechanism that causes inhibition or loss of stimulation of Na-K-ATPase by high concentrations of ANG II is not clearly understood. Studies from our laboratory and others have shown that ANG II abolishes the stimulation of Na-K-ATPase via NO-cGMP signaling in rat renal proximal tubules (1, 15). Here, we also studied the role of NO signaling in ANG II-mediated Na-K-ATPase regulation by treating the proximal tubules with the NOS inhibitor l-NAME in SHR and WKY rats. Our results show that l-NAME treatment resulted in ANG II-induced stimulation of Na-K-ATPase in proximal tubules of WKY rats. We did not observe any effect of l-NAME treatment on ANG II-induced Na-K-ATPase stimulation in SHR. Interestingly, after l-NAME treatment ANG II produced similar stimulation of Na-K-ATPase in proximal tubules of SHR and WKY rats. These results suggest that ANG II via NO signaling could cause a loss of Na-K-ATPase stimulation in WKY rats, and this pathway might be defective in SHR. However, reports from other laboratories have shown that ANG II-mediated inhibition of sodium transporters involves 6-epoxy-eicosatrienoic acid, PKC, or decreased Na-K-ATPase turnover. Also, it has been shown that increased production of peroxynitrite could be involved in ANG II-mediated Na-K-ATPase inhibition. Although we did not study these pathways, it is possible that differences in cell/species type or experimental design could modulate ANG II-mediated Na-K-ATPase regulation. The present study was performed in isolated proximal tubules from SHR, while others have used rabbit proximal tubular cultures or ventricular myocytes (8, 14, 19, 23). To further investigate the effect of ANG II on NO production, we determined NO levels in ANG II (10⁻⁶ M)-treated proximal tubules of SHR and WKY rats. Our results show that ANG II treatment increased NO production in WKY rats, and this increase was sensitive to an AT1R antagonist. However, ANG II failed to increase NO levels in proximal tubules of SHR. These results suggest that NO production in response to ANG II is defective in SHR and could be responsible for continued ANG II-induced Na-K-ATPase stimulation.

One of the mechanisms that could reduce NO production is NOS uncoupling. It is known that uncoupled NOS produces superoxides instead of NO. To investigate whether NOS is uncoupled in proximal tubules of SHR, we determined NO production in SHR and WKY rats. We used rabbit proximal tubular cultures or ventricular myocytes (8, 14, 19, 23). To further investigate the effect of ANG II on NO production, we determined NO levels in ANG II (10⁻⁶ M)-treated proximal tubules of SHR and WKY rats. Our results show that ANG II treatment increased NO production in WKY rats, and this increase was sensitive to an AT1R antagonist. However, ANG II failed to increase NO levels in proximal tubules of SHR. These results suggest that NO production in response to ANG II is defective in SHR and could be responsible for continued ANG II-induced Na-K-ATPase stimulation.
the NOS inhibitor 1-NAME, suggesting NOS uncoupling in SHR. ANG II failed to produce L-arginine-mediated superoxides in proximal tubules of WKY rats. A previous study from our laboratory showed that ANG II via NADPH oxidase activation leads to superoxide production, which in turn causes NOS uncoupling (1). We found that treatment of proximal tubules with ANG II caused NADPH oxidase activation in both SHR and WKY rats. However, ANG II-induced NADPH oxidase activation was significantly higher in SHR compared with WKY rats. Interestingly, pretreatment of proximal tubules with the NADPH oxidase inhibitor DPI prevented ANG II-induced, NOS-dependent superoxide production in SHR. Furthermore, DPI treatment in SHR also normalized NO production in response to ANG II. These data suggest that inhibition of NADPH oxidase decreases superoxide production and restores NOS coupling in SHR. We speculate that in SHR an increased ANG II-induced NADPH oxidase activation could be contributing to NOS uncoupling. The uncoupled NOS not only fails to produce NO but could also increase superoxide production, which could further decrease NO bioavailability. Therefore, we believe that inhibition of NADPH oxidase could reduce superoxide levels and prevent NOS uncoupling.

To investigate the mechanism for higher stimulation of Na-K-ATPase at low concentrations of ANG II and increased ANG II-induced NADPH oxidase activation in proximal tubules of SHR, we determined AT1R protein expression and ANG II-induced G protein coupling. We found similar AT1R protein levels in proximal tubules of SHR and WKY rats. Interestingly, our data show that there was an increase in ANG II-induced AT1R-G protein coupling in SHR compared with WKY rats, which may have contributed to enhanced Na-K-ATPase and NAPDH activation in SHR.

In conclusion, our results show that micromolar concentrations of ANG II failed to activate Na-K-ATPase in WKY rats, yet in SHR it produced stimulation of Na-K-ATPase. Compared with WKY rats, we observed higher ANG II-induced NADPH oxidase activation, which may have led to NOS uncoupling and a decrease in ANG II-induced NO production in SHR. Defective ANG II-induced NO signaling could result in stimulation of Na-K-ATPase even at higher ANG II concentrations, which could lead to increased sodium reabsorption and subsequently contribute to development of hypertension.

GRANTS

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

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.A.J., M.F.L., and A.A.B. provided conception and design of research; A.A.J. performed experiments; A.A.J. analyzed data; A.A.J. interpreted results of experiments; A.A.J. prepared figures; A.A.J. drafted the manuscript; A.A.J., M.F.L., and A.A.B. edited and revised the manuscript; A.A.J., M.F.L., and A.A.B. approved final version of the manuscript.

REFERENCES


