Angiotensin II counteracts the effects of cAMP/PKA on NHE3 activity and phosphorylation in proximal tubule cells

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ABSTRACT

Binding of angiotensin II (Ang II) to the AT1 receptor (AT1R) in the proximal tubule stimulates NHE3 activity through multiple signaling pathways. However, the effects of Ang II/AT1R-induced Gi activation and subsequent decrease in cAMP accumulation on NHE3 regulation are not well established. We therefore tested the hypothesis that Ang II reduces cAMP/PKA-mediated phosphorylation of NHE3 on serine 552 and, in doing so, stimulates NHE3 activity. Under basal conditions, Ang II stimulated NHE3 activity but did not affect PKA-mediated NHE3 phosphorylation at serine 552 in opossum kidney (OKP) cells. However, in the presence of cAMP-elevating agent forskolin (FSK), Ang II blocked FSK-induced NHE3 inhibition, reduced intracellular cAMP concentrations, lowered PKA activity and prevented the FSK-mediated increase in NHE3 serine 552 phosphorylation. All effects of Ang II were blocked by pretreating OKP cells with the AT1R antagonist losartan, highlighting the contribution of the AT1R/Gi pathway in Ang II-mediated NHE3 upregulation under cAMP-elevating conditions. Accordingly, Gi inhibition by pertussis toxin treatment decreased NHE3 activity both in vitro and in vivo and, more importantly, prevented the stimulatory effect of Ang II on NHE3 activity in rat proximal tubules. Collectively, our results suggest that Ang II counteracts the effects of cAMP/PKA on NHE3 phosphorylation and inhibition by activating the AT1R/Gi pathway. Moreover, these findings support the notion that NHE3 dephosphorylation at serine 552 may represent a key event in the regulation of renal proximal tubule sodium handling by Ang II in the presence of natriuretic hormones that promote cAMP accumulation and transporter phosphorylation.

KEYWORDS: sodium transport, Na⁺/H⁺ exchanger isoform 3, AT1 receptor, inhibitory G protein, pertussis toxin
LIST OF ABBREVIATIONS and ACRONYMS

Ang II – Angiotensin II
AT1R – Angiotensin II type 1 receptor
cAMP – 3',5'-cyclic adenosine monophosphate
CIP - Calf intestinal alkaline phosphatase
FSK – Forskolin
Gi – Inhibitory G protein
J$_{HCO3-}$ – Net bicarbonate proximal tubular reabsorption
Los – Losartan
NHE3 – Na$^+$/H$^+$ exchanger isoform 3
PKA – Protein kinase A
PS552-NHE3 – NHE3 phosphorylated at serine 552
PTX – Pertussis toxin
OKP – Opossum kidney cell line clone P
INTRODUCTION

In the kidney, approximately 70% of filtered sodium is reabsorbed by the proximal tubule (3). In this segment, the principal apical membrane pathway for sodium reabsorption is Na\(^+\)/H\(^+\) exchanger isoform 3 (NHE3) (7, 8, 40, 49), which mediates the influx of extracellular Na\(^+\) into proximal tubular cells in exchange for the efflux of intracellular H\(^+\) into the tubular fluid. As such, NHE3 plays an essential role in regulating extracellular fluid volume, blood pressure control and acid-base balance. Not surprisingly, NHE3 function is under exquisite neurohormonal control (7, 8, 40, 49, 53). Among the stimuli or conditions that upregulate NHE3 are angiotensin II (Ang II) (23, 24) and the sympathetic nervous system (26, 47), whereas parathyroid hormone (PTH) (13), dopamine (28) and glucagon-like peptide-1 (GLP-1) (15, 18) act by inhibiting NHE3-mediated Na\(^+\)/H\(^+\) exchange.

The effects of Ang II on NHE3 have been extensively studied (17, 35, 37, 56), and several studies have identified a number of signal transduction pathways involved in NHE3 regulation by Ang II. Ang II stimulation of NHE3 activity in proximal tubule cells is thought to depend at least in part on pathways typically triggered by Gq protein activation, namely, PKC and IP\(_3\)/Ca\(^{2+}\) signaling. Low-dose Ang II-mediated upregulation of NHE3 was associated with PKC translocation and was abolished by PKC inhibition (31). Moreover, Banday and Lokhandwala (5) observed that AT1R upregulation under oxidative stress conditions promotes NHE3 activation in proximal tubules via a PLC/Ca\(^{2+}\)-calmodulin (CaM)-dependent mechanism. Finally, IRBIT (IP\(_3\)R binding protein released with inositol 1,4,5-trisphosphate), which is released from the IP\(_3\) receptor into the cytosol by higher levels of the competitive ligand IP\(_3\) (2), is also involved in the activation of NHE3 by Ang
II, together with changes in intracellular Ca\(^{2+}\) and CaM-dependent protein kinase II-dependent pathway (25).

However, although the binding of Ang II to AT1R has also been reported to activate Gi signaling (36, 46, 50, 55), which in turn inhibits cAMP generation and, consequently, reduces protein kinase A (PKA) activation (1, 4, 38), whether and how Gi activation and subsequent cAMP decreases triggered by AT1R/Ang II regulates NHE3 activity are poorly understood. Currently, contradictory reports suggest that AT1R-mediated NHE3 activation may or may not depend on adenylate cyclase inhibition by Gi-mediated signaling (10, 38), and NHE3 phosphorylation status was not assessed in either of these studies. Considering that NHE3 is inhibited by a variety of agonists that evoke cAMP formation and PKA activation (13, 15, 28, 34) and that phosphorylation of the NHE3 C-terminal region by PKA at serine 552 is physiologically regulated both \textit{in vitro} and \textit{in vivo} (32, 33, 58), the present study aimed to address this issue further by analyzing, in a straightforward way, whether Ang II affects NHE3 activity and phosphorylation status by inhibiting the cellular responses induced by cAMP-elevating conditions in the renal proximal tubule. The results presented herein demonstrate that Ang II downregulates the cAMP/PKA signaling pathway in the renal proximal tubule and lowers NHE3 phosphorylation at serine 552, a marker for inactive NHE3 (6, 21, 33). Moreover, we have found that the stimulatory effect of Ang II on NHE3 activity is abolished by acute proximal tubular perfusion of pertussis toxin \textit{in vivo}, supporting the notion that Gi signaling activation by Ang II is an important step in regulating NHE3 under physiological conditions.
METHODS

Reagents and antibodies. Reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. The monoclonal antibody (mAb) raised against NHE3, clone 3H3, was kindly provided by Dr. Daniel Biemesderfer and Dr. Peter Aronson from Yale University (New Haven, CT). The phosphospecific anti-NHE3 antibody directed against phospho-serine 552, clone 14D5 (33), was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mAb for actin (JLA20) was acquired from Merck Millipore (Billerica, MA). The rabbit polyclonal antibody that recognizes PKA phosphorylated substrates at serine/threonine (pSer/Thr PKA) (22) was purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell culture. The opossum kidney cell line (OKP) (passages 6-12) with proximal tubular characteristics, was kindly provided by Dr. Orson W. Moe (University of Texas Southwestern Medical Center) and maintained in 75-cm² tissue culture flasks in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were incubated at 37°C in a humidified 95% air–5% CO₂ atmosphere. Cells were subcultured with phosphate-buffered saline (PBS) and 0.25% trypsin-EDTA. Every 2 days, the medium was replaced. In all experiments, cells were seeded onto tissue culture plates, grown to confluence, and serum starved for 24 h.

Rats. Animal procedures and protocols (090/15) were in accordance with the ethical principles in animal research of the Brazilian College of Animal Experimentation and were
approved by the institutional animal care and use committee. Experiments were performed using male Wistar rats (300-350 g) housed under standardized conditions (constant temperature of 22°C, 12:12-h light-dark cycle, and 60% relative humidity) at the University of São Paulo Medical School animal facility.

**Intracellular pH recovery in OKP cells.** In order to assess total NHE3 activity, the rate of intracellular pH (pHi) recovery from an intracellular load was examined as previously described (11). Briefly, OKP cells were grown to confluence on glass coverslips and loaded for 5 min with 10 µM BCECF-AM in control solution (in mM: 130 NaCl, 5 KCl, 1 MgCl2, 0.8 NaH2PO4, 0.83 Na2HPO4, 1.0 CaCl2, 7 HEPES, 25 glucose, pH 7.4). BCECF-loaded cells were placed in a thermo-regulated chamber on an inverted epifluorescence microscope (Nikon, TMD) and exposed to control solution until pHi stabilization. After pHi stabilization, cells were acidified by NH4Cl prepulse for 2 min and then exposed to control solution with or without treatment. As acute regulation of NHE3 activity by Ang II in renal proximal tubule is biphasic, with low concentrations (picomolar to nanomolar) stimulating NHE3 activity and high concentrations (nanomolar to micromolar) inhibiting transporter activity (24), all experiments in this study were performed using an Ang II concentration of 10^{-10} M. At the end of each experiment, the high K^+-nigericin method (51) was used to calibrate the BCECF signal (in mM: 20 NaCl, 130 KCl, 1 MgCl2, 1 CaCl2, 5 HEPES, containing 10 µM nigericin adjusted to pH values of 7.5, 7.1, 6.5, 6.0). pHi was monitored using a 495/440 nm dual excitation ratio method with a 150 W xenon lamp. Fluorescence emission was collected at 530 nm by a photomultiplier-based fluorescence system (Georgia Instruments, PMT-400) at 1-sec time intervals. All measurements of pHi recovery rates were obtained from the first 2 min by linear regression analysis and presented as dpHi/dt (pH units/min).
**In vivo stationary microperfusion.** Rats were anesthetized by intramuscular administration of Tiletamine/Zolazepam (50 mg/kg) and Xylazine (5 mg/kg) for *in vivo* microperfusion (42). A tracheostomy was performed, and the left jugular vein was cannulated for infusion of saline containing 3% mannitol at 0.05 ml/min. The kidney was isolated using a lumbar approach, immobilized *in situ* using Ringer-agar in a Lucite cup under a microscope and adequately illuminated. The tubule was perfused using a double-barreled micropipette: one side was filled with Sudan black-colored castor oil, the other with the luminal perfusion solution colored with 0.05% FD & C green. The intratubular pH was measured as the voltage difference between the two asymmetric sides of the H⁺ ion-sensitive microelectrode: the larger barrel contained a H⁺-sensitive ion-exchange resin (Fluka Chemika, Buchs, Switzerland), and the smaller barrel contained 1 M KCl colored by FD & C green (reference barrel). Intratubular pH changes were recorded continuously in the same tubule with a microcomputer equipped with an analog-to-digital conversion board (Lynx, São Paulo, Brazil) for data acquisition and processing. Net bicarbonate reabsorption ($J_{\text{HCO}_3}$) was measured by injecting a droplet of the luminal perfusion solution between the oil columns and following the intratubular pH changes toward the steady-state level [$(\text{pH})_0$]. From the intratubular pH values measured along this curve and from systemic $P_{\text{CO}_2}$, the intratubular concentrations of $\text{HCO}_3^-$ were calculated at one-second intervals using the Henderson-Hasselbach equation. The rate of tubular acidification was expressed as the half-time ($t_{1/2}$) of the reduction of the injected $\text{HCO}_3^-$ levels to their stationary level.

$J_{\text{HCO}_3}$ was calculated from the following equation:

$$J_{\text{HCO}_3} = k \left[ (\text{HCO}_3^-)_i - (\text{HCO}_3^-)_s \right] r/2$$
where \( k \) is the rate constant of the reduction of luminal bicarbonate \([k = \ln 2/(t 1/2)]\), \( r \) is the tubule radius, and \((\text{HCO}_3^-)_i\) and \((\text{HCO}_3^-)_s\) are the concentrations of \(\text{HCO}_3^-\) at the injected level and at the stationary level, respectively.

**cAMP assay.** cAMP levels were measured using the Cyclic AMP Direct EIA Kit (Arbor Assays ®, Ann Arbor, MI) according to the manufacturer’s instructions.

**Determination of PKA activity in cell lysates.** Protein kinase A (PKA) activity was accessed using a PKA kinase activity assay (Enzo Life Science, Farmingdale, NY) according to the manufacturer’s instructions. PKA activity was also indirectly measured using a pSer/Thr PKA antibody as previously reported (19).

**SDS-PAGE and immunoblotting.** OKP cells were grown to confluence in 24-well plates and were solubilized in 200 µl per well with SDS sample buffer (2% SDS, 10% glycerol, 100 mM β-mercaptoethanol, 0.1% bromophenol blue and 50 mM Tris, pH 6.8). Each set of 3 wells received the same treatment and were mixed after solubilization. Subsequently, 25 µl of cell lysate (for immunoblotting using the pSer/Thr PKA antibody), 40 µl (for actin and PS552-NHE3) or 60 µl (for total NHE3) were loaded per lane. The samples were then separated via 7.5% SDS-PAGE. The proteins were transferred the polyvinylidene difluoride (PVDF) membranes (Millipore Immobilon-P, Bedford, MA) in a TE 62 transfer electrophoresis unit (GE HealthCare) for 8–10 h at 350 mA, 4 °C. PDVF membranes were first blocked for 1 h in blocking solution (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4), to avoid unspecific binding, followed by overnight incubation with primary antibody diluted in blocking solution. For the pSer/Thr PKA antibody, we used the blocking solution with 5% bovine serum albumin and 0.1% Tween 20 in TBS, pH 7.4. After primary antibody incubation the PVDF membranes were washed 5 times with blocking solution and incubated for 1h with the appropriate horseradish peroxidase-
conjugated secondary antibody (1: 2,000 diluted in blocking solution). After secondary antibody incubation, the membranes were washed 5 times in blocking solution, followed by an additional 2 washes in PBS (pH 7.4). The membranes were then digitized on an ImageScanner LAS 4000 mini (GE HealthCare, Little Chalfont, UK) and quantified using ImageJ software (Bethesda, MD). To show the specificity of the phosphospecific NHE3 mAb for serine 552, cell lysates were dephosphorylated before SDS-PAGE with calf intestinal alkaline phosphatase (Promega Corporation, Madison, WI).

**Statistical analysis.** Statistical analyses were performed using one-way ANOVA followed by the Tukey post hoc test or the non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test. A value of P < 0.05 was considered significant. The results are expressed as the mean ± standard error of the mean (SEM).
RESULTS

Ang II does not affect PKA-mediated NHE3 phosphorylation at serine 552 in OKP cells under basal conditions.

To evaluate whether the time-dependent stimulatory effect of Ang II on NHE3 activity under basal conditions was associated with lower levels of PKA-mediated NHE3 phosphorylation at serine 552 in OKP cells, the effect of Ang II (10^{-10} M) on NHE3 activity in OKP cells was assessed by measuring intracellular pH (pHi) recovery and NHE3 serine 552 phosphorylation after 5, 15 and 30 min of treatment. As shown in Figure 1A, Ang II did not induce a statistically significant change in NHE3 activity after 5 min of treatment (0.380 ± 0.020 vs. 0.304 ± 0.023 pH units/min; P < 0.05), but increased it at 15 (0.528 ± 0.019 pH units/min; P < 0.001) and 30 min (0.508 ± 0.022 pH units/min; P < 0.001) compared with the control (0.304 ± 0.023 pH units/min). On the other hand, as expected, 10^{-4} M Forskolin (FSK), a compound that activates adenylyl cyclase, decreased NHE3 activity at all time points.

Next, we assessed whether NHE3 upregulation by Ang II under basal conditions would lower PKA activity and therefore reduce the levels of NHE3 phosphorylation at the PKA consensus site serine 552. To this end, OKP cells were treated with 10^{-10} M Ang II for 5, 15 and 30 min. Cells were treated with 10^{-4} M FSK as a positive control for PKA activation. As seen in Figure 1B, PKA activity was unchanged by Ang II treatment. Similarly, Ang II was unable to change NHE3 phosphorylation status at serine 552 in OKP cells under basal conditions (Figure 2A-B). In contrast, FSK-treated cells showed a robust increase in PKA activity (Figure 1B) and NHE3 phosphorylation levels at serine 552 (Figure 2A-B).
Importantly, as a control to demonstrate the specificity of the phospho-NHE3 antibody, cell lysates were dephosphorylated with calf intestinal alkaline phosphatase (CIP) before SDS-PAGE. As shown in Figure 2C, the band that usually appears at 80 kDa when blots are probed with the anti-PS552-NHE3 antibody was not detected in OKP cell lysates treated with CIP.

**Ang II counteracts Forskolin-mediated cAMP generation and PKA activation in OKP cells.**

To determine whether Ang II would reduce cAMP generation and, consequently, PKA activity in OKP cells under cAMP-elevating conditions, we measured cAMP levels and PKA activity in OKP cells pre-treated for 30 min with $10^{-4}$ M FSK in the presence or absence of $10^{-10}$ M Ang II for 5, 15 or 30 min. Figure 3A shows that Ang II treatment led to a time-dependent decrease in FSK-mediated intracellular cAMP generation (74 ± 11 (for FSK alone); 48 ± 3 (for FSK + Ang II 5 min); 41 ± 12 (for FSK + Ang II 15 min) and 29 ± 5 pmol/ml (for FSK + Ang II simultaneously, i.e., 30 min)). Both phosphorylated PKA substrates (Figure 3B) and PKA activity (Figure 3C) paralleled the decrease in intracellular cAMP levels, indicating that Ang II counteracts the FSK-induced augmentation of cAMP signaling.

**Ang II counteracts Forskolin-induced PKA-mediated NHE3 phosphorylation in OKP cells.**

Next, we sought to examine whether the cAMP decreases mediated by Ang II would effectively result in lower levels of NHE3 phosphorylation at serine 552 and enhanced NHE3-mediated transport. Total NHE3 activity was assessed by pHi recovery.
assay in OKP cells pre-incubated for 30 min with $10^{-4}$ M FSK in the presence or absence of $10^{-10}$ M Ang II for 5, 15 or 30 min. Figure 4 shows that simultaneous incubation of FSK with Ang II for 30 min ($0.329 \pm 0.032$ pH units/min) completely blocked the inhibitory effect of FSK on NHE3 activity ($0.170 \pm 0.019$ vs. $0.293 \pm 0.022$ pH units/min in CTRL). As indicated in Figure 5, Ang II at both 15 min ($106 \pm 8\%$) and 30 min ($92 \pm 4\%$) incubation was also able to counteract the effect of FSK on NHE3 phosphorylation levels at serine 552 ($161 \pm 6$ vs. $100 \pm 2\%$, $P < 0.001$ vs. CTRL) (Figure 5).

Angiotensin II counteracts the effects of Forskolin on NHE3 activity and phosphorylation via the AT1 receptor in OKP cells. We performed similar functional and molecular assays to verify whether the effects of Ang II shown in Figures 2 and 4 are mediated by the AT1 receptor. Therefore, OKP cells were treated with $10^{-6}$ M losartan (Los), a specific AT1 receptor blocker, before being pre-incubated with $10^{-4}$ M FSK for 30 min in the presence or absence of $10^{-10}$ M Ang II for 5, 15 or 30 min. Los treatment blocks the effects of Ang II in counteracting the FSK-induced inhibition of NHE3 activity (Figure 6). Consistent with this result, Ang II was unable to block FSK-induced NHE3 phosphorylation at serine 552 (Figure 7). Together, these results indicate that Ang II counteracts the effects of FSK on NHE3 via the AT1 receptor.

Inhibitory G protein (Gi) blockade prevents the stimulatory effect of Ang II on NHE3 activity in renal proximal tubules of Wistar rats. To examine whether the observed effects of Ang II on FSK-induced NHE3-mediated transport and transporter phosphorylation could be explained by the activation of
an AT1R/Gi protein signaling pathway, we evaluated the effects of Ang II on NHE3 activity and phosphorylation in the presence of pertussis toxin (PTX), a Gi protein inhibitor and cAMP-elevating agent (39). First, we evaluated the role of Gi protein per se in the regulation of NHE3 activity and phosphorylation status by treating OKP cells with PTX for 5, 15 or 30 min. PTX treatment reduced NHE3 activity in OKP cells under otherwise basal conditions (Figure 8A) (0.272 ± 0.029; 0.162 ± 0.011; 0.178 ± 0.013 and 0.124 ± 0.010 pH units/min at 0 (CTRL), 5, 15 and 30 min respectively), and this decreased activity was accompanied by an increase in NHE3 phosphorylation at serine 552, (100 ± 2; 158 ± 16; 155 ± 11 and 155 ± 16% at 0 (CTRL), 5, 15 and 30 min respectively) (Figure 8B-C).

We then performed stationary in vivo microperfusion to measure the rate of bicarbonate reabsorption ($J_{\text{HCO}_3}$-) in native rat renal proximal tubules exposed to $10^{-10}$ M Ang II and/or 10 ng/ml PTX. As indicated in Figure 9, PTX per se inhibits NHE3 activity in the native renal proximal tubule (1.228 ± 0.120 vs. 2.896 ± 0.111 nmol/cm²×s, $P < 0.001$). More importantly, simultaneous perfusion of Ang II with PTX prevented the stimulatory effect of Ang II on NHE3 activity (1.650 ± 0.225 vs. 4.361 ± 0.179 nmol/cm²×s, $P < 0.001$), suggesting that Ang II induces NHE3 activation at least in part by an AT1/Gi-dependent mechanism.
DISCUSSION

In mammalian renal proximal tubules, a number of hormones regulate sodium transport by altering cellular cAMP levels. Several natriuretic hormones, including PTH (13), dopamine (28) and GLP-1 (18), increase intracellular cAMP levels, thereby activating PKA and reducing sodium reabsorption in the renal proximal tubule by increasing NHE3 phosphorylation with subsequent transporter inhibition. In the present study, we investigated whether Ang II can reduce cAMP/PKA-mediated NHE3 phosphorylation at serine 552, leading to increased NHE3-mediated transport in renal proximal tubule cells. Our results provide novel evidence that Ang II counteracts the effects of cAMP/PKA-induced phosphorylation and inhibition of NHE3 by activating AT1/Gi signaling in renal proximal tubule cells.

Many studies using different experimental models have shown that Ang II increases NHE3 activity (27, 31, 38, 48, 54), and several reports observed that the mechanisms underlying Ang II-stimulated NHE3 activity in the proximal tubule may depend on PKC (31), Ca\(^{+2}\) (5) and IP\(_3\) (25), indicating the participation of AT1R/Gq signaling. Nevertheless, it has not been clear whether the AT1R can also regulate NHE3 via Gi, which is also known to couple to AT1R. To address this issue, our experiments were designed in such a way that cAMP levels were not negligible during *in vitro* experiments, while *in vivo* analyses were conducted to directly assess the role of Gi in NHE3-mediated transport in healthy animals.

Our results show that FSK treatment decreases NHE3 activity in OKP cells by increasing PKA activity, as well as PKA-mediated NHE3 phosphorylation at the consensus site serine 552, while Ang II treatment completely reverted FSK-mediated cAMP accumulation and NHE3 phosphorylation and inhibition by an AT1R-dependent
mechanism. This result is in line with previous reports that AT1R/Ang II signaling mediates cAMP decreases in proximal tubule cells (36, 38, 46, 50) and also differs from results observed by Cano et al. (10) that Ang II has no effect on cAMP levels in OKP cells under different cAMP-elevating conditions. Considering that high-passage cells show alterations in morphology, response to stimuli and protein expression compared with low-passage cell lines (9, 57), these differences might be attributed to the fact that the previous study used OKP cells at passages 32-45, whereas we used cells at low passages, namely, 6-12. Accordingly, other works that reported cAMP decreases induced by Ang II used proximal tubule cultures at lower passages (36, 50) or even primary tissues (38, 46). With regard to cAMP levels in OKP cells under basal conditions, we did not observe an inhibitory effect induced by Ang II, but it must be taken into account that basal cAMP levels were very low in culture and that hormone-induced cAMP decreases are usually more pronounced in the presence of a stimulatory agent (30). We have demonstrated using in vitro experiments and in vivo stationary microperfusion that PTX-treated proximal tubule cells showed inhibited NHE3 activity and increased NHE3 phosphorylation, suggesting the involvement of Gi in maintaining a certain level of NHE3 activity under basal conditions both in vitro and under physiological conditions. Additionally, simultaneous perfusion of native proximal tubules with Ang II and PTX prevented the stimulatory effect of Ang II on NHE3 activity. These results suggest that Ang II may stimulate NHE3 activity at least in part through AT1/Gi pathway activation. In line with this, previous in vivo microperfusion experiments in rat proximal tubules have shown that intravenous Ang II increases bicarbonate reabsorption by decreasing tubular cAMP levels and that PTX treatment attenuates Ang II-induced increases in bicarbonate reabsorption (38). Notably, our microperfusion experiments used acute luminal treatment with both PTX
and Ang II, whereas the previous report was based on intravenous Ang II infusion and chronic PTX treatment. These experimental differences may explain why we were able to observe the complete loss of NHE3 stimulation by Ang II. Moreover, the results from our acute treatments support the notion that the physiological responses observed here are not mediated by indirect processes from chronic and/or intravenous treatments.

We and others have already observed that NHE3 function is negatively correlated to its phosphorylation status, i.e., when NHE3 activity is increased, its phosphorylation at serine 552 is decreased, and vice versa (14-16, 45, 58). Consistently, NHE3 phosphorylation by PKA seems to be necessary for its inhibition (33). Taken together, these reports suggest that fine regulation of this posttranslational mechanism could be important for modulating NHE3 function even by stimulatory agents. A recent report has suggested that NHE3-mediated increases in sodium reabsorption induced by low-frequency acute electrical stimulation of the renal nerve (ESRN) occur via intrarenal renin-angiotensin system (RAS) activation and AT1R/Gi signaling (47). This study demonstrated that the ESRN-mediated increases in NHE3-mediated sodium reabsorption are due to increased intrarenal Ang II, which in turn causes a decrease in urine cAMP levels and consistently reduces PKA-mediated NHE3 phosphorylation at serine 552. The results presented here provide direct support for the notion that Ang II is involved in cAMP/PKA signaling and NHE3 phosphorylation in the proximal tubule.

Importantly, the second messenger cAMP plays a crucial role in the regulation of several physiological functions, such as vascular permeability and salt and water transport (20, 47, 52), all of which participate in blood pressure control. Thus, it is not surprising that alterations of Gi- and cAMP-dependent signaling have been associated with various pathological conditions, including hypertension. Several abnormalities in Gi-protein
expression have been found in genetic models of hypertension, spontaneously hypertensive rats (SHR) (44), and different models of experimentally induced hypertensive rats (1, 43). Increased Gi-protein expression precedes the onset of the hypertensive state in both SHR (44) and DOCA-salt hypertensive rats (43), indicating that increases in Gi-protein expression and deregulation of intracellular cAMP levels could contribute to the pathogenesis of hypertension. Moreover, in heart failure (HF), a disease associated with water and sodium retention, extracellular volume expansion and edema, HF rats display an AT1R-dependent increase in cortical NHE3 abundance (41), and our group has demonstrated that this increase in NHE3 activity is associated with a decrease in serine 552 phosphorylation (29). Similarly, young SHR exhibit increased RAS-dependent sodium retention before the development of hypertension due to increased AT1R expression in the proximal tubule (12), and our group also observed increased NHE3 activity in young SHR compared with young normotensive Wistar Kyoto (WKY), which is associated with decreased NHE3 serine 552 phosphorylation by PKA in renal proximal tubule microvilli (14). These observations were consistent with the regulation of NHE3 phosphorylation at serine 552 by Ang II reported here.

It is important to emphasize that the present study does not rule out the involvement of Gq-dependent mechanisms and other signaling pathways in proximal tubule NHE3 upregulation mediated by Ang II. Indeed, there are numerous compelling reports indicating that these alternative pathways are effectively recruited in different contexts. Nevertheless, the present study demonstrates that Gi activation by Ang II/AT1R counteracts the effects of the cAMP/PKA-induced phosphorylation and inhibition of NHE3 in proximal tubule cells and is essential for the stimulatory effect of Ang II under conditions associated with increased cAMP levels and NHE3 phosphorylation at serine 552. Furthermore, the results
presented here suggest that Ang II modulates the net phosphorylation of proximal tubule NHE3 and, in so doing, neutralizes the cellular pathway triggered by many important natriuretic factors.
FUNDING

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Figure 1 – Effects of Ang II on NHE3-mediated transport and PKA activity in OKP cells. OKP cells grown to confluence on glass coverslips were incubated with vehicle, 10^{-10} M Ang II or 10^{-4} M Forskolin (FSK) for 5, 15 or 30 min. (A) Rates of intracellular pH recovery in OKP cells. (B) PKA activity was measured by ELISA in OKP cells lysates. Data are expressed as the means ± SEM. P values calculated using one-way ANOVA followed by the Tukey post hoc test. *P < 0.05 and ***P < 0.001 vs. CTRL. N indicated on the bars.

Figure 2 – Effects of Ang II on NHE3 phosphorylation at the PKA consensus site in OKP cells. OKP cells grown to confluence on glass coverslips were incubated with vehicle, 10^{-10} M Ang II or 10^{-4} M Forskolin (FSK) for 5, 15 or 30 min. (A) OKP cell lysates were prepared for immunoblotting and incubated with monoclonal antibody against NHE3 phosphorylated at serine 552 (PS552-NHE3, 1:1,000), NHE3 (1:1,000) and actin (1:5,000). (B) The relative abundance was quantified by densitometry, and the data are presented as the ratio between the levels of phosphorylated and total NHE3, normalized to control. (C) OKP cell lysates were prepared for immunoblotting and incubated with monoclonal antibody against NHE3 phosphorylated at serine 552 (PS552-NHE3, 1:1,000), NHE3 (1:1,000) and actin (1:5,000). To confirm the specificity of the PS552-NHE3 band, cell lysates were treated (+) or not (-) with 1 U/µl calf intestinal alkaline phosphatase (CIP) before SDS-PAGE. Data are expressed as the means ± SEM. P values calculated using non-
parametric Kruskal-Wallis test followed by the Dunn's post hoc test. **P < 0.01 vs. CTRL.

N indicated on the bars.

Figure 3 – Time-dependent effect of Ang II on the cAMP/PKA signaling pathway and on PKA substrate phosphorylation in OKP cells exposed to Forskolin. OKP cells grown to confluence on glass coverslips were treated for 30 min with 100 µM Forskolin (FSK) or vehicle in the presence or absence of 10^{-10} M Ang II for 5, 15 or 30 min. (A) cAMP levels were measured by ELISA, and (B) 25 µl cell lysate were prepared for immunoblotting with antibody against PKA phosphorylated substrates (1:1,000). The relative abundance was quantified by densitometry, and the data presented as PKA activity relative to control. (C) PKA activity was measured by ELISA. Data are expressed as the means ± SEM. P values calculated using one-way ANOVA followed by the Tukey post hoc test. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. CTRL; #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. FSK. N = 4 experiments.

Figure 4 – Opposing effects of Ang II and Forskolin on NHE3 activity in OKP cells. Intracellular pH recovery rates in OKP cells grown to confluence on glass coverslips treated for 30 min with 100 µM Forskolin (FSK) or vehicle in the presence or absence of 10^{-10} M Ang II for 5, 15 or 30 min. Data are expressed as the means ± SEM. P values calculated using one-way ANOVA followed by the Tukey post hoc test. **P < 0.01 and ***P < 0.001 vs. CTRL; ##P < 0.01 vs. FSK. N indicated on the bars.

Figure 5 – Opposing effects of Ang II and Forskolin on NHE3 phosphorylation levels in OKP cells. (A) OKP cells grown to confluence on glass coverslips were treated for 30
min with 100 µM Forskolin (FSK) or vehicle in the presence or absence of $10^{-10}$ M Ang II for 5, 15 or 30 min. OKP cell lysates were prepared for immunoblotting and incubated with monoclonal antibody against NHE3 phosphorylated at serine 552 (PS552-NHE3, 1:1,000), total NHE3 (1:1,000) and actin (1:5,000). (B) The relative abundance of phosphorylated NHE3 was quantified by densitometry and presented as the percentage of NHE3 phosphorylation at serine 552 relative to control. Data are expressed as the means ± SEM. P values were calculated using non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test. ***P < 0.001 vs. CTRL and ###P < 0.001 vs. FSK. N = 6 experiments.

Figure 6 – Effects of AT1 receptor blockade on NHE3 activity in OKP cells treated with Forskolin and/or Ang II. Rates of intracellular pH recovery in OKP cells grown to confluence on glass coverslips treated for 30 min with vehicle, 100 µM Forskolin (FSK) or vehicle and/or $10^{-10}$ M Ang II for 5, 15 or 30 min in the presence of $10^{-6}$ M losartan (Los). Data are expressed as the means ± SEM. P values calculated using one-way ANOVA followed by the Tukey post hoc test. *P < 0.05 and **P < 0.01 vs. CTRL. N indicated on the bars.

Figure 7 – Effect of AT1 receptor blockade on NHE3 phosphorylation levels in OKP cells treated with Forskolin and/or Ang II. (A) OKP cells grown to confluence on glass coverslips were treated for 30 min with 100 µM Forskolin (FSK) or vehicle and/or $10^{-10}$ M Ang II for 5, 15 or 30 min in the presence of $10^{-6}$ M losartan (Los). OKP cells lysates were prepared for immunoblotting with monoclonal antibody raised against NHE3 phosphorylated at serine 552 (PS552-NHE3, 1:1,000), total NHE3 (1:1,000) and actin.
The relative abundance of phosphorylated NHE3 was quantified by densitometry and presented as the percentage of NHE3 phosphorylation at serine 552 relative to control. Data are expressed as the means ± SEM. P values were calculated using non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test. *P < 0.05 vs. CTRL. N = 6 experiments.

Figure 8 – Effects of pertussis toxin on NHE3 activity and on NHE3 phosphorylation at the PKA consensus site in OKP cells. OKP cells grown to confluence on glass coverslips were incubated with vehicle or 10 µg/µl pertussis toxin (PTX) for 5, 15 or 30 min. (A) Intracellular pH recovery rates in OKP cells. (B) OKP cell lysates were prepared for immunoblotting with monoclonal antibodies raised against phosphorylated NHE3 at serine 552 (PS552-NHE3; 1:1,000), NHE3 (1:1,000) and actin (1:5,000). (C) The relative abundance was quantified by densitometry and presented as the percentage of serine 552 phosphorylation relative to the control. In (A) P values calculated using one-way ANOVA followed by the Tukey post hoc test, whereas in (C) P values were calculated using non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test. Data are expressed as the means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. CTRL. N = 5 experiments.

Figure 9 – Effects of pertussis toxin on NHE3 activity in male Wistar rat renal proximal tubules. Bicarbonate reabsorption (J\text{HCO}_3^-) was evaluated by stationary microperfusion and continuous measurement of luminal pH in the absence or presence of 10^{-10} M Ang II and/or 10 ng/ml PTX. The number of perfused tubules is indicated in the bars. Data are expressed as the means ± SE. P values calculated using one-way ANOVA followed by the Tukey post hoc test. ***P < 0.001 vs. CTRL and ###P < 0.001 vs. Ang II.
Figure 1

A

B
Figure 2

C

<table>
<thead>
<tr>
<th>80 kDa</th>
<th>PS552-NHE3</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 kDa</td>
<td>NHE3</td>
</tr>
<tr>
<td>42 kDa</td>
<td>Actin</td>
</tr>
</tbody>
</table>

CTRL  | CTRL  | Ang II 5' | Ang II 15' | Ang II 30' | FSK 5' | FSK 15' | FSK 30' |

(-)   | ( + ) CIP
Figure 3

A

![Bar chart showing cAMP levels](image)

B

![Western blot analysis](image)
Figure 3
Figure 4

![Graph showing dpH/dt (pH units/min) for different treatments. CTRL, FSK, FSK + Ang II 5', FSK + Ang II 15', FSK + Ang II 30', Ang II. The graph compares the dpH/dt values with error bars, indicating statistical significance with ** and *** symbols.](image-url)
Figure 5

A

80 KDa –
PS552-NHE3

80 KDa –
NHE3

42 KDa –
Actin

CTRL  FSK  FSK + Ang II 5'  FSK + Ang II 15'  FSK + Ang II 30'  Ang II

B

![Bar chart showing PS552-NHE3/NHE3 (% CTRL) for various conditions.](chart.png)

CTRL  FSK  FSK + Ang II 5'  FSK + Ang II 15'  FSK + Ang II 30'  Ang II

***  ***  ###  ###  # # #

PS552-NHE3/NHE3 (% CTRL)
Figure 6

The bar chart shows the change in pH units per minute (dpH/dt) for various conditions. The conditions tested include:

- CTRL
- FSK + Los
- FSK + Los + Ang II 5'
- FSK + Los + Ang II 15'
- FSK + Los + Ang II 30'
- Ang II + Los
- Los

Significant differences are indicated by * (p < 0.05) and ** (p < 0.01). The number of observations for each condition is noted below each bar.
Figure 7

A

![Western Blot Image]

- 80 KDa
- PS552-NHE3
- 80 KDa
- NHE3
- 42 KDa
- Actin

- CTRL
- FSK + Los
- FSK + Los + Ang II 5'
- FSK + Los + Ang II 15'
- FSK + Los + Ang II 30'
- Ang II + Los
- Los

B

![Bar Graph Image]

- PS552-NHE3/NHE3 (% CTRL)

- CTRL
- FSK + Los
- FSK + Los + Ang II 5'
- FSK + Los + Ang II 15'
- FSK + Los + Ang II 30'
- Ang II + Los
- Los

* Significance (*)
Figure 9

[Bar graph showing the comparison of J_{HCO3^-} (nmol/cm^2.s) across different conditions: CTRL, Ang II, PTX, Ang II + PTX. Each bar is labeled with a number (15, 25, 19, 21) and includes error bars. Significant differences are marked with asterisks: *** for Ang II compared to CTRL, ### for PTX compared to Ang II, and *** for Ang II + PTX compared to Ang II.]