Proximal tubule NHE3 activity is inhibited by beta-arrestin-biased angiotensin II type 1 receptor signaling

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Submitted 13 March 2015; accepted in final form 3 August 2015

THE Na+/H+ EXCHANGER ISOFORM 3 (NHE3) is present in the apical membrane of the renal proximal tubule and mediates the majority of NaCl and NaHCO3 reabsorption in this nephron segment (4, 37). Given its importance in body fluid homeostasis and blood pressure whereas pharmacological concentrations of ANG II (above 10−7M) inhibit NHE3 activity. These effects are mainly driven by the AT1 receptor, a seven-transmembrane G protein-coupled receptor (GPCR).

Classically, activation of the AT1 receptor by its full agonist ANG II leads to dissociation of the heteromeric G protein into Gαi (Gαi and Gβγ) and Gβγ subunits, spreading the signal for renal sodium retention. In the proximal tubule, the signal transduction activated by ANG II involves several different mechanisms and pathways: the stimulatory effect is associated with activation of protein kinase C and/or adenyl cyclase inhibition leading to a decrease of intracellular cAMP generation (22, 29, 30). The binding of inositol 1,4,5-triphosphate receptor-binding protein (IRBIT) to the COOH-terminal of the NHE3 is also associated with increased NHE3 activity and expression in the plasma membrane and is dependent on changes in intracellular Ca2+ and Ca2+/calmodulin-dependent protein kinases II (22). On the other hand, the inhibitory effect of pharmacologic doses of ANG II is associated with the activation of phospholipase A2 and protein kinase G (2, 45).

Intriguingly, numerous studies have shown that activation of the AT1 receptor could also signal via G-protein-independent mechanisms (1, 33, 34, 43). Furthermore, some AT1 ligands have the ability to trigger one selective pathway over another and thus are referred to as biased agonists (1, 39–41). Among those are the custom-synthesized peptides, TRV120023 and TRV120027, that selectively, via AT1R, elicit beta-arrestin 1/2 signaling without any detected activation of G-protein coupling. Indeed, these compounds are unable to accumulate inositol monophosphates (IP1) or diacylglycerol (DAG) in HEK-293 cells overexpressing the AT1 receptor, whereas silencing of beta-arrestin-2 eliminates the ability of TRV120027 and TRV120023 to promote late phosphorylation of ERK, Akt, or endothelial nitric oxide (NO) synthase, well-established downstream effectors of beta-arrestin (6, 41). In a model of myocardial injury reperfusion, TRV120023 conferred cardioprotection by enhancing cardiac contractility, decreasing cell mortality, and blocking the effects of endogenous ANG II. These beneficial cardiac effects were abolished in beta-arrestin 2 knockout mice (33).

The beta-arrestin biased ligands of the AT1 receptor also promote renal actions that are distinct from those exerted by ANG II (5, 6). In dogs with acute heart failure, TRV120027...
increased urinary flow and sodium excretion associated with a decrease in fractional proximal sodium reabsorption (5). Therefore, the present study aimed to test the hypothesis that biased beta arrestin signaling through AT1 receptor inhibits NHE3 activity in the renal proximal tubule.

METHODS

Reagents and antibodies. TRV120023 was generously provided by J. D. Violin (Trevena, King of Prussia, PA), S3226 (38) was generously provided by J. Punter (Sanofi-Aventis Deutschland, Frankfurt, Germany), PD123319 was purchased from Tocris Bioscience (Bristol, UK), and EZ-Link Sulfo-NHS-SS-Biotin and immunopurope immobilized streptavidin were purchased from Thermo Fisher Scientific (Rockford, IL). The monoclonal antibody (mAb) raised against OKP cell NHE3, clone 3H3, and was kindly provided by Biebersderfer and Aronson (Yale University) (27). The mAb against beta-arrestins 1/2, clone D24H9, was purchased from Cell Signaling Technology (Danvers, MA). The polyclonal antisera NHE3-C00, used for the immunofluorescence assays, was from the McDonough Laboratory (44). The mAb to villin was from Immunotech (Chicago, IL), the polyclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Santa Cruz Biotechnology (Dallas, TX), and anti-actin antisera was from Merck. Alexa 488-conjugated goat anti-rabbit, Alexa 568-conjugated goat anti-mouse secondary antibodies, and ProLong Antifade were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated secondary antibodies were purchased from (Thermo Fisher Scientific). Polyvinyl difluoride (PVDF) membranes were purchased from Millipore Immobilon-P (Millipore, Bedford, MA). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Cell culture. The opossum proximal tubule (OKP) cells were maintained in 75-cm² tissue culture flasks in DMEM high-glucose medium Thermo Fisher Scientific supplemented with 10% (vol/vol) of heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified 5% CO₂, 95% air atmosphere. Cells were subcultured using Ca²⁺/Mg²⁺-free phosphate-buffered saline and 0.25% trypsin-EDTA. The culture medium was replaced every 2 days. For experiments, cells were subcultured in tissue culture plates, grown to confluence, and serum starved for 24 h before the studies were performed.

Animal protocols. Animal procedures and protocols were followed in accordance with the ethical principles in animal research of the Brazilian College of Animal Experimentation and National Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committees from University of São Paulo School of Medicine and University of Southern California Keck School of Medicine. In vivo microperfusion experiments were performed using male Wistar rats (220–260 g body wt) and in vivo immunofluorescence were performed on male Sprague-Dawley rats (200–250 g body wt) that were kept under diurnal light conditions and had free access to food and water.

Stationary microperfusion. Rats were anesthetized with ketamine-xylazine-acepromazine (64.9, 3.20, and 0.78 mg/kg sc, respectively) and placed on a heated surgical table to maintain body temperature. The left jugular vein was cannulated for infusion of mannitol in isotonic saline solution at a rate of 0.1 ml/min. The microperfusion procedure was performed as described previously (15). Briefly, the proximal tubule was punctured using a double-barreled micropipette. One barrel was used to inject FDC-green colored Ringer perfusion solution (in mM: 80 NaCl, 5 KCl, 25 NaHCO₃, 1 CaCl₂, 1.2 MgSO₄, and raffinose to reach isonitonic, at 0 PCO₂) and the other to inject Sudan black colored castor oil used to block the injected fluid columns in the lumen. To measure the tubular pH, a double-barreled, asymmetric microelectrode was used. The larger barrel was silanized with hexamethyldisilazane and contained an H⁺-sensitive ion-exchange resin. The smaller barrel contained a 1-M KCl reference solution colored by FDC-green. The voltage between the micro electrode barrels, representing the tubular H⁺ activity, was continuously measured by the electrode via a WPI electrometer (Sarasota, FL) and recorded by an analog to digital converter (Lynx, São Paulo, Brazil).

The rate of tubular acidification was expressed as the half time of the exponential reduction of the initial HCO₃⁻ concentration, [HCO₃⁻]initial, to its stationary level. The bicarbonate reabsorption (J[HCO₃⁻] stationary) per centimeters squared of the proximal tubule was calculated using the following equation:

\[ J_{\text{HCO}_3^-} = \frac{\ln 2}{t_{1/2}} \left[ \text{HCO}_3^- \right]_{\text{initial}} - \left[ \text{HCO}_3^- \right]_{\text{stationary}} \times \frac{r}{2} \]

where the value of \( J_{\text{HCO}_3^-} \) is in mmol·cm⁻²·s⁻¹, \( t_{1/2} \) is the acidification half time, the initial bicarbonate concentration is 25 mM, the stationary bicarbonate concentration is calculated from the stationary luminal pH and the arterial PCO₂, and \( r \) is the tubular radius.

Immunofluorescence. Rats were anesthetized intraperitoneally with Inactin (110 mg/kg) and a small dose of intramuscular ketamine (100 μl). Body temperature was maintained thermostatically at 37°C. Polyethylene catheters (PE-50) were inserted into the carotid artery to monitor blood pressure and into the jugular vein for infusion of drugs and 4% BSA in 0.9% saline at 40 μl/min to maintain euvolement. Blood pressure was measured continuously and remained within the autoregulatory range (80–110 mmHg). At the completion of all surgical procedures, the animals were allowed to equilibrate for 15 min before infusion of drugs. Rats were either infused with 4% BSA in 0.9% saline (control) or infused with TRV120023 (50 μg·kg⁻¹·min⁻¹) in the same BSA-saline solution for 20 min. At the end of each treatment, the left kidneys were fixed in situ by removing the capsule and placing the isolated kidney in a small Plexiglas cup and bathing it in PLP fixative (2% paraformaldehyde, 75 mM lysine, and 10 mM Na-periodate, pH 7.4) for 5 min to avoid changes in perfusion pressure. The kidneys were then removed, cut in half in a midsagittal plane, and postfixed in PLP fixative (2–4 h). The fixed tissue was rinsed with PBS, cryoprotected by overnight incubation in 30% sucrose in PBS, embedded in Tissue-Tek OCT Compound, and frozen in liquid nitrogen. Cryosections (5 μm) of TRV120023 treated and paired control were cut and transferred to charged glass slides and air dried. For immunofluorescence labeling, the sections were rehydrated in PBS for 10 min, followed by a 10-min wash in 50 mM NH₄Cl in PBS and antigen retrieval with 1% SDS in PBS for 5 min. After two 5-min washes in PBS, the sections were then blocked with 1% BSA in PBS to reduce background. Double-labeling was performed by incubating with polyclonal antisera NHE3-C00 and monoclonal antibody against villin, both at a dilution of 1:100 in 1% BSA in PBS for 2 h at room temperature. After three 5-min washes in PBS, the sections were incubated with a mixture of Alexa 488-conjugated goat anti-rabbit and Alexa 568-conjugated goat anti-mouse secondary antibodies diluted 1:500 in PBS, mounted in ProLong Antifade, and dried overnight at room temperature. Slides were viewed with a Zeiss LSM 510 confocal system with differential interference contrast overlay and microscopy. Results shown are representative of results in three sets of rats assayed.

Na⁺-dependent intracellular pH recovery by fluorescence microscopy. NHE3 activity was measured in OKP cells as the rate of Na⁺-dependent intracellular pH (pHᵢ) recovery after an acid load with NH₄Cl (in mM: 20 NH₄Cl, 125 NaCl, 5 KCl, 1 MgCl₂, 0.83 NaH₂PO₄, 0.83 NaHPO₄, 1 CaCl₂, 8 HEPES, and 25 mM glucose, pH 7.4) as previously described (9). pHᵢ was monitored by dual ion-specific ion-exchange microelectrodes (AJP-Cell Physiol; • doi:10.1152/ajpcell.00072.2015 • www.ajpcell.org).
coverslips loaded 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, and 25 mM glucose, pH 7.4) for 5 min and placed in a thermoregulated chamber mounted on an inverted epifluorescence microscope (TMD; Nikon). After several washes, the BCECF-loaded cells were exposed to control solution until pH stabilization and then prepulsed with NH4Cl for 2 min for subsequent acid loading. After acid load, cells were exposed to control solution or TRV120023, losartan, telmisartan, S2326, and PD123319 diluted in control solution and the rates of Na+-dependent pH recovery were acquired. At the end of each experiment, the high K+-nigericin method was used to calibrate the BCECF signal (in mM: 20 NaCl, 130 KCl, 1 MgCl2, 1 CaCl2, and 5 HEPES, containing 10 μM nigericin adjusted to pH values of 7.5, 7.1, 6.5, and 6.0). For all the experiments the Na+-dependent pH recovery rate was calculated from the first 2 min by linear regression analysis and presented as dpHi/dt (pH units/min).

**Silencing of beta-arrestins 1 and 2 in OKP cells.** Simultaneous silencing of beta-arrestins 1 and 2 was performed using small interference RNA (siRNA), a 19-nucleotide duplex RNA with 3'-dTdT overhangs. To design OKP cell beta-arrestins 1 and 2-specific siRNA duplexes, the cDNAs coding for these two isoforms of beta-arrestins were amplified and sequenced from OKP cells (a cell line derived from Didelphis virginiana). Briefly, total RNA was isolated from OKP cells using TRizol Reagent (Thermo Fisher Scientific) following manufacturer’s protocol and was subsequently treated with Dnase-I RNase free and purified using RNAeasy Mini Kit, Quiagen (Venlo, Limburg). Random primers were used for reverse transcription. RT-PCR was carried out using primers designed based on the beta-arrestin 1 and beta-arrestin 2 sequences from the South American oppossum Monodelphis domestica available in databases (beta-arrestin 1: XM_007490958.1; beta-arrestin 2: XM_001365861.3). The following primers were used: beta-arrestin 1: forward: 5'-TCGATGGTTGG-TTCTGGTG-3' and reverse: 5'-ACCTTAGGCGGCTGGTTC-3'; and beta-arrestin 2: forward: 5'-GGCTTCTCGATGTTGGTGC-3' and reverse: 5'-ATAAGGGAGCTTGTGCCTG-3'. Amplitcons were sequenced by Sanger method. After beta-arrestin 1 and beta-arrestin 2 cDNA sequences from OKP were determined, custom Silencer Select siRNAs and a scramble Silencer Select Negative Control #1 (5'-CAGUAGUACACUUUGUGAAU-3', sense sequence) were obtained from Dharmacon (GE Healthcare). The siRNA sense sequences against beta-arrestin 1 and beta-arrestin 2 were 5'-CAUACAGUGUUAAGUGCC-3' and 5'-GACUUUGCCUUUGAAGGAA-3', respectively. OKP cells were plated at 50% confluence and transfected with both siRNAs against beta-arrestin 1/2 transcripts (80 nM) or with the same concentration range of TRV120023 to determine the appropriate dose as well as to evaluate if TRV120023, like ANG II, exhibits a bimodal effect. As shown in Fig. 1C, TRV120023 significantly reduced the Na+-dependent pH recovery at concentrations above 10 μM and was consistently inhibitory. The pH recovery rate, in pH units/min, decreased from 0.234 ± 0.014 at baseline to 0.083 ± 0.014 and 0.094 ± 0.013 for 10-7 and 10-5 M TRV120023, respectively. The time course of inhibition of Na+-dependent pH recovery by 10-7 M TRV120023 was assessed at 2, 15, and 30 min of treatment. As observed in Fig. 1D, the activity decreased from a baseline of 0.216 ± 0.007 to 0.109 ± 0.013 at 2 min of treatment and further decreased to 0.068 ± 0.005 pH units/min at 30 min. Based on these findings, all the following experiments were conducted with 10-7 M TRV120023 for 15 min.

**Essential requirement for beta-arrestins in TRV120023-mediated inhibition of Na+-dependent pH recovery in OKP cells.** To ascertain that beta-arrestins were essential for mediating the inhibitory effects of TRV120023 on proximal tubule Na+-dependent pH recovery, beta-arrestins 1/2 were knocked down by siRNA in OKP cells. As shown in Fig. 2A, transfection of OKP cells with siRNA for beta-arrestins 1/2 (siRNA b-art) efficiently reduced beta-arrestin’s expression by ~60%. Silencing of beta-arrestins 1/2 per se did not affect the Na+-
dependent pH recovery rate [0.211 ± 0.019 vs. 0.193 ± 0.025 pH units/min in OKP cells transfected with siRNA scramble (siRNA scr Ctrl); Fig. 2B]. On the other hand, the inhibitory effect of TRV120023 on Na+-dependent pH recovery rate (0.083 ± 0.017 pH units/min in siRNA scr TRV120023) was completely abolished by siRNA b-arr. In concert, these results indicate that beta-arrestins 1 and/or 2 are required for proximal tubule Na+-dependent pH recovery inhibition by TRV120023.

**Beta-arrestin-biased AT1 receptor signaling inhibits NHE3 activity in native renal proximal tubule.** Since Na+-dependent pH recovery in an indirect measure of NHE3 activity, we evaluated if these measurements were affected by 15-min pretreatment with the NHE3 specific inhibitor S3226 at 10^{-6} M (9, 11). As seen in Fig. 3A, the S3226 insensitive component of Na+-dependent pH recovery was not affected by TRV120023 in OKP cells leading to the conclusion that it is the S3226-sensitive component that is inhibited by TRV120023.

To determine whether beta-arrestin-biased AT1 receptor signaling inhibits NHE3 activity in vivo, stationary microperfusion was performed in native rat renal proximal tubule. As shown in Fig. 3B, TRV120023 decreased net bicarbonate reabsorption (\(J_{\text{HCO}_3}\)) 27%, from 2.001 ± 0.082 to 1.530 ±

![Fig. 1. TRV120023 decreases Na\(^{+}\)−dependent pH recovery rates in proximal tubule opossum proximal tubule (OKP) cells. Na\(^{+}\)−dependent pH recovery measurements in OKP cells treated with vehicle or TRV120023. Representative Na\(^{+}\)−dependent pH recovery curve from vehicle [control (Ctrl); A] and 10^{-7} M TRV120023 (B). C: Na\(^{+}\)−dependent pH recovery of OKP cells treated with 10^{-11}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M of TVR120023 for 15 min (n = 7). D: OKP cells treated with 10^{-7} M TRV120023 for 2, 15, and 30 min (n = 7). Data expressed as means ± SE (*P < 0.05, **P < 0.01, and ***P < 0.001 vs. Ctrl).](http://ajpcell.physiology.org/)

![Fig. 2. Beta-arrestins are required for proximal tubule Na\(^{+}\)−dependent pH recovery inhibition by TRV120023. A and B: efficacy of beta-arrestins knocked down by siRNA in OKP cells. Confluent OKP cells transfected with either siRNA scramble (siRNA scr) or siRNA for beta-arrestins 1/2 (siRNA b-arr) were treated with vehicle or 10^{-7} M TRV120023 for 15 min. A: cell lysates were subjected to SDS-PAGE, transferred to a PVDF membrane, and incubated with a primary antibody against beta-arrestins 1/2 (1:1,000) and subsequently with an antibody against GAPDH (1:10,000). B: relative abundance of beta-arrestins 1/2 was quantified by densitometry and normalized to GAPDH. Data expressed as means ± SE (***P < 0.001 vs. siRNA scr; n indicated in bars). C: Na\(^{+}\)−dependent pH recovery measurements in OKP cells transfected with siRNA scr or siRNA b-arr were treated with vehicle or 10^{-7} M TRV120023. Data expressed as means ± SE (***P < 0.001 vs. siRNA scr (Ctrl) and #P < 0.05 vs. siRNA scr (TRV); n indicated in the bars).](http://ajpcell.physiology.org/)
0.108 nmol·cm$^{-2}$·s$^{-1}$. In agreement with the in vitro studies the S3226-insensitive component of NHE3 activity was also not affected by TRV120023 in vivo native rat proximal tubule (Fig. 3B). Taken together these results support the conclusion that TRV120023 inhibits NHE3 activity in renal proximal tubule.

TRV120023 modulation of NHE3 activity is mediated by AT$_1$ receptor activation. Previous studies have shown that TRV120023 effects are mediated via the AT$_1$ receptor (33, 39). To confirm that the TRV120023 inhibitory effect on proximal tubule NHE3 activity is due to AT$_1$ receptor activation, Na$^+$-dependent pH$_i$ recovery was assessed in OKP cells pretreated with the AT$_1$ receptor antagonist losartan (10$^{-6}$ M) or the AT$_2$ receptor antagonist PD123319 (10$^{-6}$ M) in the presence or absence of TRV120023. As summarized in Fig. 4, the AT$_2$ receptor antagonist had no effect on the inhibition of NHE3 activity by TRV120023 while the AT$_1$ receptor antagonist prevented TRV120023-mediated inhibition of OKP cell NHE3 activity. These results show that TRV120023-mediated inhibition of NHE3 is dependent on AT$_1$ receptor activation and independent of the AT$_2$ receptor.

Beta-arrestin-biased AT$_1$ receptor signaling blunts the stimulatory effect of ANG II on NHE3 activity in renal proximal tubule. Since we confirmed that the TRV120023 effects were due to AT$_1$ receptor activation in the proximal tubule cells, we tested the hypothesis that TRV120023 blocks ANG II stimulation of NHE3 activity. To address this aim, Na$^+$-dependent pH$_i$ recovery was measured in OKP cells pretreated with 10$^{-10}$ M ANG II for 15 min followed the addition of TRV120023 for another 15 min. As seen in Fig. 5A, TRV120023 completely reverses the stimulatory effect of ANG II on NHE3 activity (0.260 ± 0.013 pH units/min) to control (0.196 ± 0.016 pH units/min). Likewise, in native rat renal proximal tubule, microperfusion of TRV120023 reverses the stimulatory effect of ANG II on NHE3 activity (3.001 ± 0.212 vs. 2.057 ± 0.088 nmol·cm$^{-2}$·s$^{-1}$; Fig. 5B). Together, these findings demonstrate that beta-arrestin-biased AT$_1$ receptor signaling triggered by TRV120023 blunts the stimulatory effect of ANG II on proximal tubule NHE3 activity.

Comparison between the effects of TRV120023 and ANG II receptor blockers on NHE3 activity. Previous studies suggested that TRV120023/TRV120027 could provide additional beneficial effects compared with the gold-standard therapeutic angiotensin receptor blockers (ARBs) (5, 24, 39). Thus we investigated if the AT$_1$ receptor antagonists losartan and telmisartan were able to exert any local tonic effect on NHE3 activity, like TRV120023. To address this question, Na$^+$-dependent pH$_i$ recovery in OKP cells and stationary microperfusion in native proximal tubule in the presence or absence of losartan and/or telmisartan were performed. As summarized in
Fig. 6, Na⁺-dependent pHᵢ recovery rates were unaffected by the presence of either losartan or telmisartan. However, a reduction in the net bicarbonate reabsorption was observed with losartan treatment (2.00 ± 0.08 to 1.74 ± 0.06 J_HC₃⁻ nmol·cm⁻²·s⁻¹), representing a decrease of 13%. These results show that TRV120023 exerts a more profound inhibitory effect on NHE3 activity compared with ANG II receptor blockers.

**TRV120023 effects on subcellular distribution of proximal tubule NHE3.** The distribution of NHE3 along the microvillar domains in vivo or decrease and increase in NHE3 expression on the surface of the membrane in vitro are usually associated with the activity of the exchanger (3, 44). To address if the inhibition of NHE3 activity was due to a diminished expression of the exchanger in cell surface membranes, cell surface protein biotinylation was performed in OKP cells. As seen in Fig. 7A, TRV120023 treatment reduced the expression of the NHE3 protein at the surface membrane by 43 ± 9% compared with vehicle treated cells. As expected, 15-min exposure of OKP cells to TRV120023 did not alter the total cellular amount of NHE3 (Fig. 7B). These results suggest that TRV120023 mediates NHE3 inhibition in OKP cells via modulation of NHE3 subcellular localization.

To investigate if acute TRV120023 treatment in vivo provoked redistribution of renal proximal tubule NHE3 from the body of the microvilli where it is active to the base of the microvilli where activity is inhibited (10), in situ immunofluorescence was performed after an acute infusion of TRV120023. The microvillar domain was labeled with a monoclonal antibody to the microvillar actin bundling protein villin (red) and a polyclonal anti-NHE3 antibody (in green). As seen in Fig. 8, acute TRV120023 infusion leads to a clear retraction of proximal tubule NHE3 from the body to base of the microvillar domain. These findings indicate that TRV120023-mediated inhibition of NHE3 activity may be the result of the retraction of the exchanger to the base of the microvillar domain in proximal tubule, a mechanism associated with decreased NHE3 activity (7, 10, 17, 27, 32).

**DISCUSSION**

Besides the classical role of beta-arrestins in promoting G protein-coupled receptors internalization and desensitization, recent evidence has shown that beta-arrestins 1 and 2 can also activate specific signal pathways in a G protein-independent manner leading to distinct cellular responses (35, 39, 43). In this study, we investigated the acute effect of beta-arrestin-biased AT₁ receptor signaling on NHE3 activity in renal proximal tubule. To this end, we used the ANG II synthetic analog TRV120023, which belongs to a new class of pharmaco-
ological agents (39). TRV120023 activation of beta-arrestin-biased AT1 receptor signaling decreases blood pressure, increases urine flow rate and sodium excretion and decreases fractional sodium reabsorption in the proximal tubule of healthy canines and those with heart failure (5, 6). Herein, we extend those findings to the cellular and molecular levels to demonstrate that TRV120023 inhibits NHE3 activity in a proximal tubule cell line as well as in the native rat proximal tubule. The results suggest that the diuretic, natriuretic, and antihypertensive effects exerted by TRV120023 may be attributed to, at least in part, inhibition of proximal tubule NHE3.

Divergent functional actions of AT1 and AT2 receptors have been reported with respect to blood pressure and sodium transport: ANG II AT1 receptor activation increases blood pressure and sodium retention whereas AT2 activation lowers blood pressure and increases sodium excretion (8, 20). A reduction in bicarbonate reabsorption mediated by AT2 receptor activation has been reported in rabbit proximal tubule cultured cells (19), suggesting that activation of the ANG II-initiated AT2 signaling cascade leads to NHE3 inhibition. Our data indicate that the inhibitory effect of TRV120023 on NHE3 activity occurs through AT1 receptor activation and does not involve the activation of AT2 receptors. These results are in line with previous studies that demonstrated that these AT1 receptor-biased agonists display a remarkable specificity for the AT1 receptor (39). Moreover, our findings suggest that besides the opposing physiological effects found between the AT1 and AT2 receptors, the activation of G-protein vs. beta-arrestin signaling of the AT1 receptor can also lead to opposite effects with respect to NHE3 modulation, thereby adding an additional level of complexity to the regulation of NHE3-mediated NaCl and NaHCO3 reabsorption proximal tubule. Interestingly, our results suggest that beta-arrestin-biased AT1 receptor signaling by TRV120023 exerts only inhibitory effects on proximal tubule NHE3 activity, which contrasts with the bimodal effect observed by the full agonist ANG II.

In OKP cells, the inhibitory effect of TRV120023 on the proximal tubule NHE3 activity was accompanied by a 43% decrease in the surface expression of NHE3, which is in the same order of magnitude as the decrease in the NHE3 activity (~45%). In addition, indirect immunofluorescence in native proximal tubule showed a clear and rapid retraction of the NHE3 from the top to the base of the microvillar domain. These findings indicate that subcellular redistribution of NHE3 plays a key role in the observed inhibition of the NHE3 activity by the beta-arrestin-biased AT1 receptor signaling. In fact, the association between redistribution of NHE3 between the brush border membranes and changes on NHE3 function has been reported repeatedly in the literature (10, 17, 23, 32). In this regard, a recent mathematical model for NHE3-mediated Na+ reabsorption predicted that NHE3 redistribution to the base of the microvillar domain creates cytosolic alkaline pH microdomains (7). The predicted effect was supported in vivo by demonstrating alkaline pH microdomains and that NHE3 activity was reduced by ~32%. These findings corroborate a previous model that suggested that NHE3 is pH sensitive and predicted that NHE3 would sharply turn off in conditions of cellular alkalosis (42).

Pharmacological inhibition of the renin-angiotensin system (RAS) is widely used in the treatment of patients with chronic renal failure and cardiovascular disorders, including hypertension and heart failure. Clinical studies are now underway to assess the efficacy and safety of the biased agonist of the AT1 receptor TRV120027 to treat acute heart failure (14). It remains to be established whether biased agonism of the AT1 receptor may indeed provide additional beneficial effects compared with ARBs. Similar to ARBs, TRV120023/TRV120027 block the pressor effect of the AT1 receptor, but unlike ARBs,
TRV120023/TRV120027 are capable of unloading the heart while preserving renal function (33, 39). These benefits were associated with the selectivity and potency to evoke beta-arrestin recruitment, which were absent in the ARB treatment (33, 39). In the present study, we show that the biased agonist TRV120023 exerts a tonic inhibitory effect on NHE3 activity both in vitro and in vivo. Conversely, neither losartan nor telmisartan inhibits NHE3 activity in OKP cells whereas by in situ microperfusion stationary experiments losartan caused a 13% reduction of NHE3-mediated bicarbonate reabsorption. The main conclusion from our experiments (under our conditions) is that proximal tubular perfusion/incubation with the biased AT1 agonist TRV120023 seems to be much more effective in inhibiting NHE3 transport activity than losartan.

Gurley et al. (18) emphasized the importance of proximal tubule sodium transport in blood pressure control by demonstrating that selectively deleting proximal tubule AT1 receptors decreases blood pressure ~10 mmHg. Those mice demonstrated improved pressure-natriuresis against ANG II-dependent hypertension associated with a significant downregulation of NHE3. The pressure natriuresis mechanism is the central feedback system for control of blood pressure, whereby increases in renal perfusion pressure lead to a decrease in renal sodium reabsorption. Interestingly, the redistribution of NHE3 between the microvillar microdomains of the apical membrane of the proximal tubule plays a crucial role in the pressure natriuresis response (10, 32). Several studies have attempted to identify the intrarenal mechanisms that could explain the interplay among hypertension, NHE3 redistribution, and pressure natriuresis (25, 26). These studies suggest that high renal perfusion pressure induces the production of NO and metabolites by the endothelial cells and that diffusion of NO to the proximal tubule cells may induce a redistribution of NHE3 to the base of the microvilli, inhibiting NHE3-mediated proximal tubule sodium reabsorption and consequently increasing natriuresis. In favor of this hypothesis, systemic inhibitors of the NO synthase decrease the natriuretic effect induced by the acute increase in the blood pressure (13, 31, 36). Recent findings have shown the beta-arrestin-biased AT1 receptor signaling may be involved in the mechanotransduction of shear stress to intracellular signals and NO production by endothelial cells (35). It is therefore tempting to speculate that activation of the beta-arrestin-biased AT1 receptor signaling may play a role in pressure natriuresis by regulating NHE3 subcellular distribution in the proximal tubule.

In summary, our data provide the first evidence that activation of the AT1 receptor/beta-arrestin signaling leads to proximal tubule NHE3 inhibition associated with subcellular redistribution of the exchanger. The modulation of NHE3 by RAS is mediated by a myriad of molecular mechanisms and numerous signaling pathways. Our results bring another player to the complexity of NHE3 regulation in renal proximal tubule and raise the question of whether biased signaling through beta-

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**Fig. 8.** Effect of beta-arrestin-biased AT1 receptor signaling on microvillar domain localization of NHE3 in native proximal tubule. Indirect immunofluorescence microscopy of the NHE3 redistribution in rats infused for 20 min with vehicle (left) or TRV120023 (right). Different sets of experiments were conducted using anti-NHE3 (1:100) detected with the secondary antibody AlexaFluor 568 (green) and anti-villin (1:100) detected with the secondary antibody AlexaFluor 488 (red). Bar = 20 μm.
arrestin-biased AT1 receptor signaling is physiologically active in the renal proximal tubule.

GRANTS

This work was supported by the São Paulo Research Foundation (FAPESP) Grants 2012/10146-0, 2013/50384-0, and 2013/16019-8 (to A.C.Girardi) and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-083785 (to A. A. McDonough).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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