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Regulation of Na^+/H^+ exchanger-NHE3 by angiotensin-II in OKP cells

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Abstract

Previous studies have shown that circulating Angiotensin II (A-II) increases renal Na^+ reabsorption via elevated Na^+/H^+ exchanger isoform 3 (NHE3) activity. We hypothesized that prolonged exposure to A-II leads to an increased expression of renal NHE3 by a transcriptionally mediated mechanism. To test this hypothesis, we utilized the proximal tubule-like OKP cell line to evaluate the effects of 16-h treatment with A-II on NHE3 activity and gene expression. A-II significantly stimulated NHE3-mediated, S-3226-sensitive Na^+/H^+ exchange. Inhibition of transcription with actinomycin D abolished the stimulatory effect of A-II on NHE3-mediated pH recovery in acid-loaded OKP cells. This prolonged exposure to A-II was also found to elevate endogenous NHE3 mRNA (by 40%)—an effect also abolished by inhibition of gene transcription. To evaluate the molecular mechanism by which A-II regulates NHE3 expression, the activity of NHE3 promoter driven reporter gene was analyzed in transient transfection assays. In transfected OKP cells, rat NHE3 promoter activity was significantly stimulated by A-II treatment, and preliminary mapping indicated that the A-II responsive element(s) is present between 149 and 548 bp upstream of the transcription initiation site in the NHE3 gene promoter. We conclude that a transcriptional mechanism is at least partially responsible for the chronic effects of A-II treatment on renal NHE3 activity.

Keywords: Slc9A3; Proximal tubule; Kidney; Antiport; Activity; Promoter

1. Introduction

Angiotensin-II (A-II) is an essential hormone that exerts pleiotropic actions in the renal proximal tubule including modulation of transport [1,2], metabolism [3], and cell proliferation [4]. A-II is a powerful vasoconstrictor and strong mediator of intravascular volume regulation [5]. A-II also regulates NaCl reabsorption [5,6] through both direct effects on the proximal tubule, and indirectly via effects on aldosterone secretion. A-II stimulates Na^+ uptake in isolated proximal tubule cells through elevations in the activity of an amiloride-sensitive Na^+/H^+ exchanger (NHE) which could be inhibited by A-II receptor antagonist saralasin [7]. Subsequently, a wide range of evidence has been acquired showing that the specific NHE isoform NHE3 is acutely regulated by A-II [8,9]. The acute regulation of NHE activity on the renal proximal tubules is biphasic; low concentrations of A-II (10^{-11} M) have been shown to stimulate Na^+/H^+ antiport activity, whereas high concentrations (10^{-7} M) inhibit NHE activity utilizing distinct signaling pathways [10–12]. A-II regulates NHE3 activity after binding to membrane AT1 receptors in the proximal tubules [5,13]. More recent studies have shown that the increase in NHE3 activity induced by acute A-II treatment involves stimulating protein kinase C [8], activating a non-receptor tyrosine kinase [11], or redistributing of NHE3 protein [14,15].

The effect of chronic A-II treatment on NHE3 expression and the regulation of salt and water balance is less clear. A recent in vivo study showed that long-term A-II treatment increased the abundance of NHE3 in the thick ascending limb and in the proximal tubule brush border, an effect which may contribute to the observed enhancement of renal Na^+ and HCO_3 reabsorption in response to A-II [16]. Our group has also

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demonstrated that increased renal NHE3 activity coincides with significantly elevated NHE3 protein and mRNA abundance in rats chronically infused with A-II via implanted miniosmotic pump [17]. These findings suggest that prolonged exposure to elevated levels of A-II increases NHE3 activity which may result partially from a transcriptional response of NHE3 gene.

Previous work has demonstrated that the proximal tubule-like OKP cells provide an excellent model system to study in situ regulation of the NHE3 gene [18–20]. The OKP cell line, established from opossum kidney proximal tubule, expresses NHE3 in a similar fashion to that observed in the proximal tubule apical membrane [18]. For this reason, OKP cells have been used as a model for understanding hormonal regulation of NHE transporter expression. In OKP cells, NHE expression and activity are elevated by endothelin, acidosis, and glucocorticoids, and are inhibited by PTH and exogenous cAMP analogs [19,21–23]. The effects of A-II on NHE in these cells have not been investigated. In the present study, we utilized OKP cells to evaluate A-II mediated changes in NHE expression, and to determine the molecular mechanisms by which A-II may exert its effects on NHE3 promoter activity. In these studies, we demonstrate that long-term A-II treatment (16 h) induces transcriptionally mediated (actinomycin D-sensitive) increase in NHE3-driven pH recovery, paralleled by a significantly elevated NHE3 mRNA abundance and its gene promoter activity. These results suggest a role for A-II in transcriptional regulation of the NHE3 gene.

2. Methods

2.1. Cell culture

OKP cells, a gift from Dr. R. J. Alpern, are a clonal opossum kidney cell line, originally described by Cole et al. [24]. OKP cells (passages 21–30) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The same batch of FBS was used in all experiments. Cells were cultured at 37 °C in a 95% air–5% CO_2 atmosphere and passaged every 48–72 h. For experimentation, OKP cells were grown to confluence, rendered quiescent by removing the serum for 48 h, then studied. In A-II treatment experiments, cells were incubated with 100 nM (10^{-7} M) A-II (Sigma; St. Louis, MO) or vehicle (saline) for 16 h. A-II is stable for 24 h–48 h in cell culture [25,26]. In all experiments, control and experimental cells were from the same passage and were assayed on the same day. Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA).

2.2. Measurement of NHE activity

NHE activity was analyzed by measuring the rate of intracellular pH (pH_i) recovery after induction of an acid load in the absence of $NaHCO_3$. pH_i was assessed by monitoring the fluorescence emission of the pH-sensitive dye SNARF-1 (Molecular Probes, Eugene, OR). For these experiments, cells were grown on no. 1 glass coverslips and incubated with 5 μM SNARF1-AM for 20 min at 37 °C in a 5% CO_2-equilibrated incubator. Cells were washed once with HEPES Buffered Saline Solution (HBSS) and incubated in HBSS for 10 min at 37 °C to allow for full de-esterification of the dye. The HBSS contained (in mmoles/liter): 5 KCl, 0.3 KH_2PO_4, 138 NaCl, 0.3 Na_2HPO_4, 10 HEPES, 1.3 $CaCl_2$, 0.2 $NaHCO_3$, 0.4 $MgSO_4$, 5.6 glucose, and 5 glutamine, pH 7.4).

Coverslips containing SNARF-1 loaded cells were mounted in a temperature-regulated and media perfused chamber and transferred to the microscope stage. The perfusion system provides complete media exchange within 5 s. An Olympus IX-70 microscope equipped with a 40×, 1.4 NA objective was used to acquire light emitted from the cells excited by 525 nm light from a 100 W Hg source. A Multispectral Imager (Optical Insights; Albuquerque, NM) was used to split the emission beam, forming side-by-side images of emitted light at 640 nm (basic pH wavelength) and 570 nm (acidic pH wavelength) that were acquired on a single charge coupled device imaging array (Photometrics; Tucson, AZ) [27]. The ratio of fluorescence intensity (640 nm/570 nm) was measured for individual cells, and these ratios were subsequently converted to pH_i by means of an in situ-derived calibration curve [28]. The relation between the SNARF fluorescence ratio and pH_i was fitted using the following equation: $pH = pK + \log[(R - R_{min})/(R_{max} - R)]$, where R represents the experimentally measured ratio of 640 nm/570 nm, R_{min} is the ratio measured at the most acidic pH, and R_{max} is the ratio measured at the most basic pH. At the conclusion of each experiment, R_{min} and R_{max} were assessed for each individual cell by adding media containing nigericin and valinomycin in high K^+ [28] and measuring the ratio at pH 6.6. and 8.2, respectively. This procedure allows for normalization of estimated pH between individual cells. The in situ derived pK_a value for SNARF-1 was 7.6±0.1, $n=3$.

For acid loading, cells were incubated in the presence of 20 mM NH_4Cl for 5 min, and then the perfusion medium was switched to an NH_4Cl-free, HCO_3-free HBSS. Upon removal of external NH_4Cl, NH_3 diffuses from the cells, leaving an intracellular acid load. The pH_i recovery rate is assessed by monitoring the pH_i change in the first 1 min after NH_4Cl is replaced with NH_4Cl/HCO_3-free HBSS. To dissect the contribution of different isoforms of NHE to the initial rate of H^+ efflux, the rate of pH_i change recorded in the presence of 20 μM HOE-694, which inhibits both NHE1 and NHE2, and 20 μM S3226 was used to assess the specific activity of NHE3 [29,30]. In addition, recovery from the acid load was assessed during inhibition of H^+-ATPase with 0.4 μM bafilomycin A1 [31].

2.3. RNA purification and Northern blot analyses of OKP cells

mRNA was isolated from three different passages of OKP cells, utilizing the Fast-Track mRNA purification kit (Invitrogen, Carlsbad, CA). A ^{32}P-labeled OKP cell-specific NHE3 cDNA antisense probe was generated using strip-EZ PCR kit (Ambion, Austin, Texas), according to the manufacturer's protocol. The opossum NHE3 cDNA, which was used as a template for making cDNA antisense probe, was produced by RT-PCR with primer pairs designed and synthesized based on GenBank (accession No. L42522). The primer sequence was as follows: 5′-CTACATCATCGCACTCTGGA-3′ (sense) and 5′-TCAC-CAGAGACAGAAGGAAG-3′ (antisense), which produced an OKP cell cDNA from +453 bp to +1114 bp. The 662 bp PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and excised and purified from the gel. This PCR-generated product was confirmed by DNA sequencing. 5 μg of mRNA was fractionated on 1.0% formaldehyde-agarose gels, transferred onto nylon membranes (Pierce), and cross-linked to the membrane by ultraviolet irradiation. The filter was hybridized with the probe overnight at 42 °C in 50% formamide containing hybridization buffer and washed under high stringency conditions (0.1× SSC–0.1% SDS at 42 °C). β-actin-specific cDNA antisense probes were used as internal standards for quantitating NHE3 gene expression.

2.4. Semiquantitative RT-PCR analysis of NHE3 gene expression

mRNA was purified from OKP cells treated for 16 h with A-II (100 nM) or vehicle (saline). RT-PCR conditions were described with standard methods [32]. The primers used to detect NHE3 were designed from OKP NHE3 mRNA (GeneBank accession no. L42522). The forward primer was at 453–472 bp (5′-CTACATCATCGCACTCTGGA-3′), and the reverse primer was at 1095–1114 bp (5′-CTTCCTTCTGTCTCTGGTGA-3′). The expected amplicon size from NHE3 mRNA is 662 bp. The primers used to detect β-actin were purchased from Stratagene (La Jolla, CA). The expected amplicon size from the β-actin gene is 661 bp. Subsaturation levels of cDNA templates needed to produce a dose-dependent amount of PCR product were defined in initial experiments by testing a range of template concentrations. Subsequent PCR was carried out with subsaturation levels of RT reactions with identical amplification parameters. PCR was performed with OKP NHE3 or β-actin primers in separate reactions, and then equal volumes of both PCR reactions were loaded on the same gel and visualized with ethidium bromide. The optical density of each band was determined by Quantity One software (Bio-Rad, Hercules, CA).

Table 1
Primer sequence used for PCR amplification of the rat NHE3 promoter

No.	Construct	Primer sequence
1	−548/+39	5′-GACTACGCGTCCTCTTCCCATCCTATGTCC-3′ (forward)
2	−149/+39	5′-GACTACGCGTACCAAGTAGGTGGGCGTGA-3′ (forward)
3	−95/+39	5′-GACTACGCGTTGTTTGCATTCACGTGCG-3′ (forward)
4	−548/+39	5′-TACACTCGAGTCACAGCCCTCTGCGCGCTAT-3′ (reverse)

NHE3 mRNA expression levels were estimated by taking a ratio of NHE3 over β-actin amplicon optical densities.

2.5. Construction of reporter plasmids

Luciferase reporter plasmids used in this study were derived from pGL3/basic (pGL3b; Promega), which contains the firefly luciferase gene. A construct containing −1360/+58 bp of the rat NHE3 promoter was described previously [33]. The other constructs (−548/+39, −149/+39, −95/+39) were prepared by PCR amplication using the −1360/+58 bp construct as a template, primer 4 as a common reverse primer and primers 1, 2, and 3 as forward primers (Table 1). For these constructs, PCR products were digested with *MluI*/*Xho*I and subcloned into pGl3-basic vector, at the *MluI*/*Xho*I sites. All plasmid constructs were confirmed by sequencing on both strands.

2.6. Transient transfection and functional promoter analysis

OKP cells (at passages 21–30) were cultured in 24-well plates. When cells reached ~70–80% confluence, liposome-mediated transfection was performed as follows: 0.5 μg of promoter construct DNA (constructs mentioned above), 30 ng of pRL-CMV (*Renilla* luciferase reporter construct used as an internal standard; Promega), and 5 μl of Lipofectamine (GIBCO/BRL; Grand Island, NY) were mixed with 200 μl of Opti-MEM (GIBCO/BRL) for 30 min at room temperature. Then the mixture was added to the cells, the cells were incubated for 5 h, and then an equal volume of DMEM containing 20% FBS was added. On the next day, the medium was removed and replaced with standard medium with 10% FBS. Twenty-four hours later, cells were harvested for reporter gene assays. For A-II dose response studies, transiently transfected cells were treated with 0, 10^{-10}, or 10^{-7} M A-II for 16 h before they were harvested. For transcriptional inhibition studies, transiently transfected cells were pretreated with actinomycin D (100 nM; Calbiochem-Novabiochem; San Diego, CA) for 2 h and then treated with 100 nM A-II for 16 h in the presence of actinomycin D before they were harvested. Promoter reporter assays were performed using the Dual Luciferase Assay Kit according to the manufacturer's instructions (Promega). Luciferase activities were measured with a tube luminometer (FB12; Zylux; Maryville, TN). Each experiment was repeated a minimum of three times with different passages of cells.

2.7. Statistical analyses

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

3. Results

3.1. The effect of A-II concentration on NHE3 promoter activity in OKP cells

Previous studies on A-II mediated acute responses have shown that lower concentration of A-II (10^{-10} M) stimulates NHE3 activity, while higher A-II concentration (10^{-7} M) inhibits NHE3 activity. To test if these concentrations used for acute regulation have similar effect, NHE3 promoter constructs transfected OKP cells were treated with different concentrations of A-II for 16 h before analyzing the promoter activity. As shown in Fig. 1, lower concentration of A-II (10^{-10} M), which acutely stimulates NHE3 activity, had no effect on stimulating NHE3 promoter activity. High concentration of A-II (10^{-7} M), which has been shown to inhibit NHE3 activity in the acute setting stimulated NHE3 gene promoter activity. As a control, pGL3 B vector showed no change in all A-II concentrations used in the experiments.

3.2. Effects of chronic A-II treatment on functional NHE activity in OKP cells

To measure the NHE activity in OKP cells, the initial rate of recovery of intracellular pH (pH_i) following an acid load was measured in the presence of 0.4 μM bafilomycin A (to inhibit the V-type H^+-ATPase), and the absence of medium $NaHCO_3$. NHE3-specific activity was assessed by further addition of 20 μM HOE-694 to inhibit NHE1 and 2, and S-3226 to inhibit NHE3 specifically [31]. Bafilomycin by itself had no significant effect on pH_i recovery rate, whereas 20 μM HOE-694 reduced the pH recovery rate by about 37% compared to control (Fig. 2). The pH recovery rate measured in the presence of both bafilomycin and 20 μM HOE694 was not significantly different than recovery measured with 20 μM HOE694 alone. To specifically evaluate NHE3 activity, recovery rates were measured in the presence of both bafilomycin and 20 μM S-3226. 20 μM S-3226 reduced the pH recovery rate by about 60% compared to control (Fig. 2). No significant recovery was observed with all inhibitors

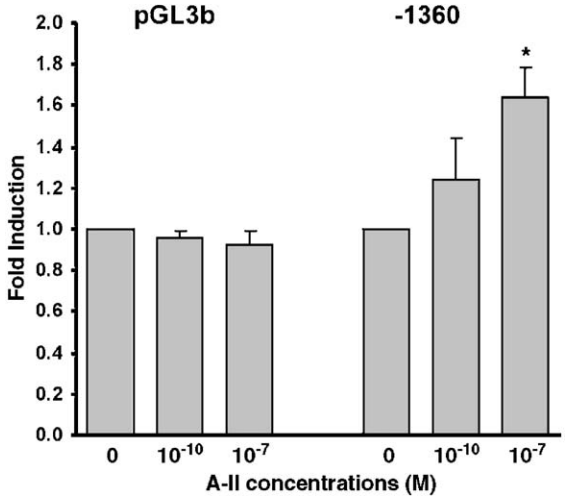

Fig. 1. Dose effect of A-II on NHE3 Gene Promoter Activity in OKP cells. OPK cells were transiently transfected with NHE3 gene promoter construct plus pRL-CMV, and were treated with different concentration of A-II (0, 10^{-10}, or 10^{-7} M) for 16 h. Data are presented as relative luciferase activity (firefly luciferase activity driven by the NHE3 gene promoter over renilla luciferase activity driven by the CMV promoter). Values are means±S.E.; number of replicates=3–5. *Statistical difference for 10^{-7} M A-II vs. all other concentrations.

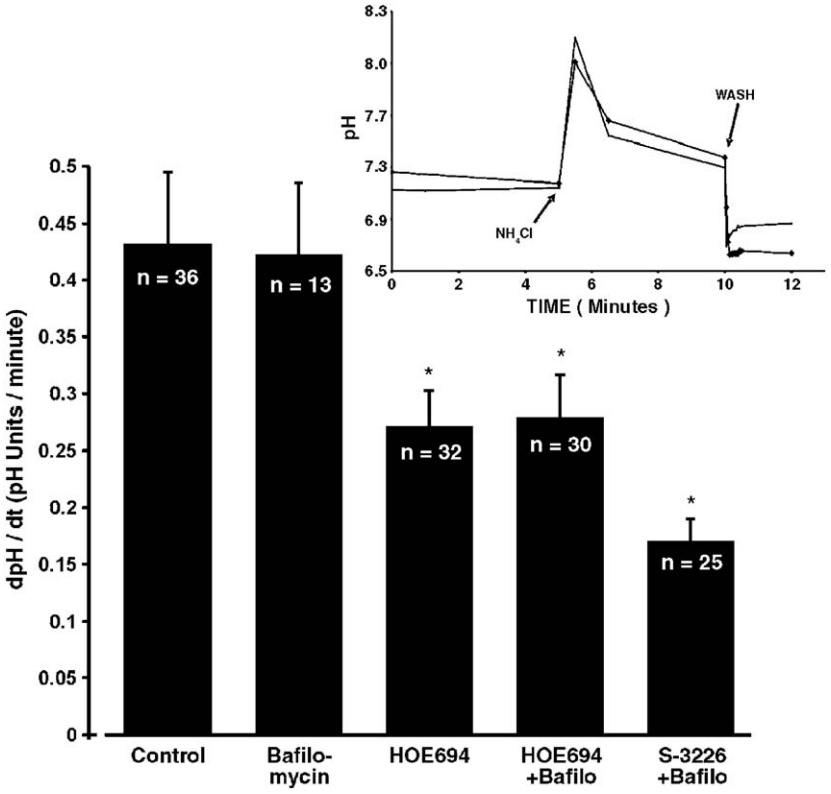

Fig. 2. Contributions of the V-type H^+-ATPase, NHE1 and NHE2 and NHE3, to the initial rate of recovery from an acid load in OKP cells. An NH_4Cl (20 mM) washout technique was used to impose an instantaneous acid load on the OKP cell cytosol [31]. Bars show the mean rates of recovery (±S.E.) measured within the first minute after NH_4Cl washout. Values presented within the bars (n=13–36) indicate number of cells analyzed. Bafilomycin A (0.4 μM) was used to inhibit the V-type H^+ ATPase, and 20 μM HOE-694 was used to inhibit the combined activities of NHE1 and 2. S-3226 was used to specifically inhibit NHE3. The drugs were added 5 min prior to initiation of the acid load. *Significantly different than control (leftmost bar). The insert demonstrates the time course for analysis of pH recovery after an acid load. Cells were loaded with NH_4Cl for 5 min, and a rapid acidification was then elicited by switching to an NH_4Cl-free medium (Martinez-Zaguilan et al., 1999). Both experiments shown were carried out in a HCO_3-free medium. Data were acquired every 3 s during the recovery phase. The top line is data averaged from 7 cells incubated without inhibitors present. The bottom line (filed circles) is the average response from 8 cells incubated in the presence of 20 μM S-3226 and 20 μM HOE694. These inhibitors were added 1 min prior to addition of NH_4Cl and were present in the wash solution as well. Recovery rates were measured over the initial 6 s after a pH minima was measured for each experiment. Recovery rates for these experiments were 0.18±0.08 pH units per second for control and 0.01±0.04 pH units per second in the presence of inhibitors. Error bars are not shown for clarity.

present (not shown). These data indicate that NHE transport (NHE1-3) is the only process for pH_i recovery from an acid load in the absence of $NaHCO_3$ and also suggest that the activity remaining in the presence of 20 μM HOE694 is due to NHE3. In a second series of experiments, the effect of 16 h of treatment with 10^{-7} M A-II on purported NHE3 mediated transport activity was evaluated by measuring pH recovery in the presence of bafilomycin and 20 μM HOE694. A-II treatment led to a doubling of NHE3 mediated transport activity (Fig. 3). To evaluate if transcription of new NHE3 was required for this A-II induced increase in activity, cells were preincubated with transcriptional inhibitor actinomycin D. Actinomycin D blocked the A-II mediated increase in HOE694-insensitive activity, indicating that A-II mediates changes in NHE3 activity through a transcriptional mechanism. The dependence of the A-II induced increase in HOE694-insensitive recovery on functional NHE3 activity was directly evaluated using S-3226. Treatment with S-3226 reversed the A-II induced rate of recovery (Fig. 3), suggesting that the A-II mediated increase was specifically dependent on an increase in NHE3 activity.

3.3. A-II increases NHE3 mRNA abundance in OKP cells

Since the observed increase in NHE3 activity was sensitive to a transcriptional inhibitor, actinomycin D, we next evaluated the effects of 16 h treatment with A-II on endogenous expression of NHE3 mRNA in OKP cells by Northern blots. Hybridization with opossum NHE3-specific cDNA probes showed that NHE3 mRNA abundance was increased by ~44% in A-II-treated OKP cells, as compared to controls (2.70±0.09 densitometric units in A-II treated cells, versus 1.88±0.05 in controls; n=3) (Fig. 4). To demonstrate that the effect of A-II on NHE3 gene expression was due to transcriptional regulation, OKP cells were first treated with actinomycin D for 2 h and then treated with A-II for 16 h in the presence of actinomycin D. Due to prolonged exposure (18 h) to transcriptional inhibitor actinomycin D, we were not able to obtain enough mRNA to perform Northern blot experiments. Therefore NHE3 mRNA abundance was determined by semiquantitative RT-PCR with OKP-specific NHE3 and β-actin primers (Fig. 5A). Preliminary experiments established standard curves that related amount of RT-reaction used for PCR to amount of amplified product, and all subsequent

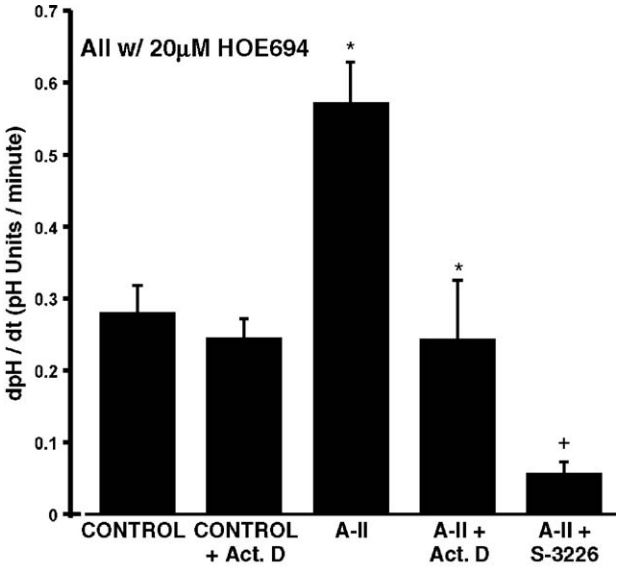

Fig. 3. Effect of Chronic A-II Treatment on Specific NHE3 Functional Activity. Bars show the mean rates of recovery (±S.E.) measured within the first minute after NH_4Cl washout from 25 to 30 cells. All measurements were performed in the presence of Bafilomycin A (0.4 μM) and 20 μM HOE-694. The remaining rate of recovery measured in the presence of both inhibitors is used as an estimate of the NHE3 functional activity (NHE-3 Specific). Act. D-100 nM actinomycin D. S-3226 at 20 μM. The drugs were added 5 min prior to initiation of the acid load. AII-16 h pretreatment with 100 nM A-II. *Significantly different than recovery measured in the absence of A-II (leftmost bar). †Significantly different than recovery measured in the absence of A-II pretreatment alone (middle bar).

experiments were carried out within the linear portions of these curves. Results showed that A-II treatment increased NHE3 mRNA abundance by ~42%, and this increase was abolished by actinomycin D treatment (1.38±0.03 densitometric units,

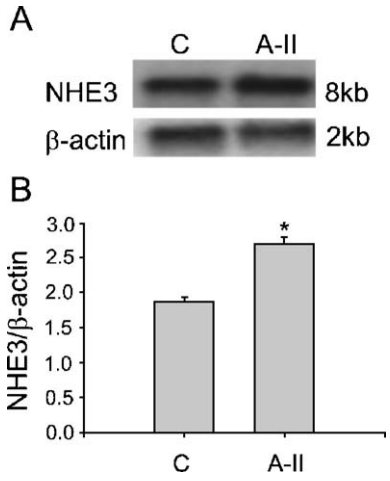

Fig. 4. Northern blot analysis of NHE3 mRNA expression in OKP cells. (A) A representative Northern blot experiment. Blots were probed with opossum NHE3-specific and β-actin-specific probes. The hybridization signal at 8 kilobase pairs (kb) represents NHE3, and hybridization signal at 2 kb represents β-actin. C—control, A-II—angiotensin-II (both panels). (B) Quantitative summary from NHE3 Northern blot experiments. Data are presented as a ratio of NHE3 to β-actin mRNA levels. Values are means±S.E.; $n=3$. *Indicates statistical significance compared to control.

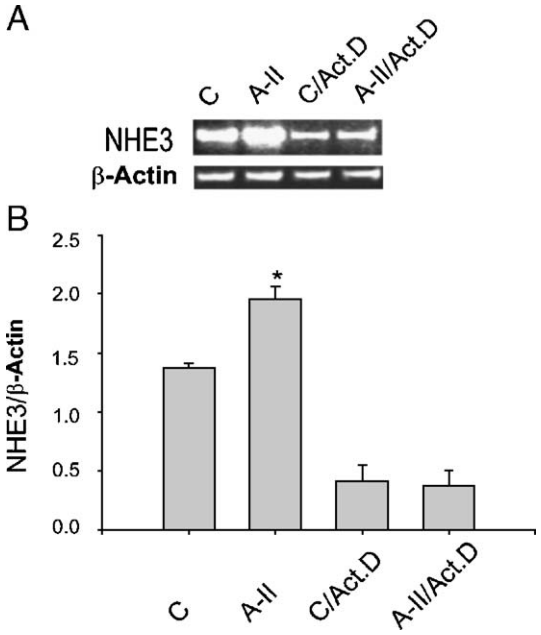

Fig. 5. Effect of actinomycin D (Act.D) on NHE3 mRNA expression in A-II-treated OKP cells. (A) Representative results of semi-quantitative RT-PCR analysis of NHE3 mRNA. mRNA isolated from OKP cells treated under different conditions was used for first-strand cDNA synthesis. Subsequent PCR was performed with subsaturation levels of the RT reaction, and NHE3 or β-actin primers were used in separate reactions. Equal volumes of PCR reactions for NHE3 and β-actin were loaded on the same gel and visualized with ethidium bromide. (B) Quantitative summary from NHE3 semiquantitative RT-PCR experiments. Data are presented as a ratio of NHE3 mRNA to β-actin mRNA levels. Results are means±S.E. from 3 to 4 separate experiments. The results showed that the ~42% increase in NHE3 mRNA abundance induced by A-II treatment was abolished by Act.D treatment (100 nM, for 18 h). *Indicates statistical significance compared to control.

control; 1.96±0.1, A-II-treated, $n=4$; 0.42±0.13, actinomycin D alone; 0.38±0.13, A-II-treated in the presence of actinomycin D; $n=3$) (Fig. 5B).

3.4. Rat NHE3 gene promoter analysis in OKP cells

To determine the A-II responsive region in the rat NHE3 gene promoter, four reporter constructs (pGL3/−1360 bp, pGL3/−548 bp, pGL3/−149 bp, pGL3/−95 bp) and the promoterless pGL3-basic were transfected into OKP cells. Reporter gene assays were performed 40 h after transfection. The luciferase assay data showed that each of these promoter constructs was functional in OKP cells. Compared with pGL3/basic controls, these promoter constructs resulted in eighteen to twenty-six-fold stimulation of reporter gene activity (Fig. 6A; $n=3$–6).

To test the effect of A-II on rat NHE3 gene promoter activity, OKP cells were transiently transfected with promoter constructs and then treated with 10^{-7} M A-II or saline for 16 h before they were harvested. Activity of Renilla luciferase (from co-transfected pRL-CMV vector) was not altered by treatment with 10^{-7} M A-II, thus validating its use as an internal control (data not shown). Exposure to A-II for 16 h increased NHE3 promoter activity in cells transfected with the pGL3/−1360 bp (by 44%±5%) and the pGL3/−548 bp (by 74%±12%)

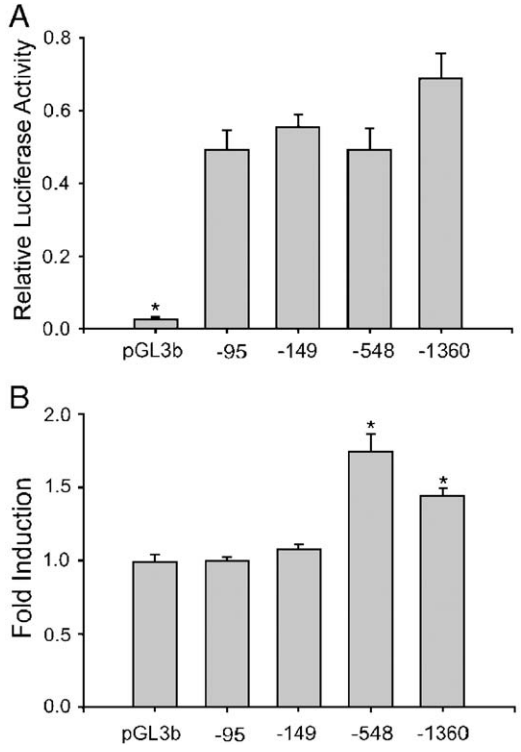

Fig. 6. Activity of rat NHE3 gene promoter in transiently transfected OKP cells. (A) OKP cells were transiently transfected with pGL3/basic (pGL3b) or NHE3 promoter constructs plus pRL-CMV. Data are presented as relative luciferase activity (firefly luciferase activity driven by the NHE3 gene promoter over renilla luciferase activity driven by the CMV promoter). Values are means ± S.E.; number of replicates = 3–5. *Statistical difference for pGL3/basic vs. all other constructs. (B) OKP cells were cotransfected with pGL3/basic or NHE3 promoter constructs plus pRL-CMV, and then cells were treated with 10^{-7} M A-II or saline for 16 h. Fold induction is shown as the ratio of luciferase activity in A-II-treated cells to that in saline treated cells. Values are means ± S.E.; number of replicates = 3–5. *Statistically different for pGL3/−548 bp and pGL3/−1360 bp vs. all other constructs.

constructs, as compared to saline treated cells. The pGL3/−95 bp and pGL3/−149 bp constructs showed no increase in promoter activity with 10^{-7} M A-II treatment (Fig. 6B).

To determine whether the A-II effect on NHE3 gene promoter activity was due to transcriptional stimulation and not altered reporter mRNA stability, OKP cells were first transfected with NHE3 promoter constructs (pGL3/−548 bp or pGL3/−95 bp) or the promoterless vector pGL3-basic and then treated with 100 nM actinomycin D for 2 h. These cells were then treated with 100 nM A-II or saline in the continued presence of actinomycin D for 16 h. Results showed that the upregulation of NHE3 promoter activity by A-II treatment in the pGL3/−548 bp construct transfected cells was blocked by actinomycin D (Fig. 7; $n = 3–5$). Furthermore, A-II did not change the reporter gene activity in cells transfected with pGL3b or pGL3/−95 bp construct regardless of the actinomycin D treatment.

4. Discussion

Previous studies demonstrated that long-term A-II treatment increases NHE3-mediated Na^+ absorption, and NHE3 protein abundance in rat renal cortex [16,17]. Studies also showed that under acute A-II treatment conditions, 10^{-10} M A-II stimulates NHE3 activity, while 10^{-7} M A-II inhibits NHE3 activity. Here, we demonstrate that 10^{-10} M A-II has no effect on NHE3 gene expression, while 10^{-10} M A-II stimulates NHE3 gene expression in OKP cells under A-II chronic treatment (Fig. 1). These results suggest that the mechanism of A-II regulation on NHE3 activity may be different between acute regulation and chronic regulation. Our data also show an A-II-induced (16 h with 100 nM A-II) increase in NHE3-specific activity in OKP cells (Fig. 3). The rate of pH_i recovery from an acid load, measured in the presence of 20 μM HOE-694 plus bafilomycin, and in the absence of HCO_3^-, was assumed to be a specific measure of NHE3 activity. This assumption is based on the fact that HOE-694 is more effective in inhibiting NHE1 and 2 ($IC_{50} = 5$ μM in PS120 cells) then NHE3 (NHE3 is unaffected at HOE-694 concentrations up to 70–100 μM) [34]. Although it is conceivable that this increase in 20 μM HOE-694-insensitive Na^+/H^+ exchange activity could be partially related to the activity of another NHE not yet described in these cells, results obtained with S-3226, a specific NHE3 inhibitor, point to NHE3 as the major mediator of A-II-mediated pH recovery (Fig. 3). Additionally, the contribution of HCO_3 coupled transporters or V-type H^+-ATPase has been ruled out by our protocols. Thus, based on analysis of H^+ recovery rates, we concluded that A-II doubled NHE3-specific functional activity. Such an increase could be due to a direct activation of the transporter itself, insertion of stored transporters into the plasma membrane, or de novo synthesis of the transporter. Our data show that actinomycin D treatment inhibits NHE3 activity induced by A-II treatment, suggesting that transcriptional regulatory mechanisms might be involved.

To test our hypothesis about transcriptional regulation of NHE3 activity by A-II, we studied endogenous NHE3 mRNA

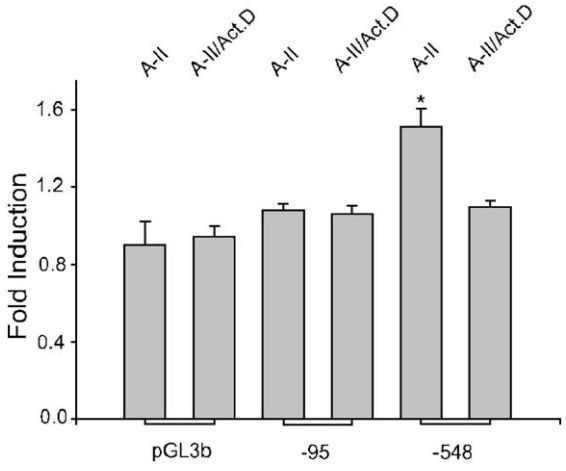

Fig. 7. Effect of actinomycin D on rat NHE3 gene promoter activity induced by A-II in transiently transfected OKP cells. Cells were cotransfected with pGL3b or NHE3 promoter constructs plus pRL-CMV. Twenty-two hours after transfection, cells were treated with 100 nM Act. D for 2 h, and then A-II or saline was applied in the presence of Act. D for 16 h before harvesting cells. Fold induction is shown as the ratio of luciferase activity in A-II-treated cells over luciferase activity in saline treated cells. Results are means ± S.E.; number of replicates = 3–5. *Statistically different for − 548/A-II vs. all other constructs and treatments.

levels in OKP. We demonstrated a significant increase in NHE3 transcript abundance with chronic A-II treatment, an effect that was abolished by inhibition of gene transcription with actinomycin D (Fig. 5). These results suggested that chronic A-II treatment stimulates NHE3 activity at least partially through gene expression regulation. In addition, A-II clearly regulated NHE3 promoter activity in cells transiently transfected with reporter gene constructs, which further supports that A-II effect on NHE3 activity involves activating NHE3 promoter. Therefore, our data suggest that chronic A-II exposure increases NHE3 activity, primarily through a transcriptionally mediated mechanism. It is interesting to note that A-II treatment elicited a similar increase in NHE3 mRNA abundance in in vivo studies in rat kidney cortex where changes in mRNA expression paralleled changes in immunoreactive protein expression [17]. To evaluate the interactions of A-II with the NHE3 gene promoter, we developed a series of reporter constructs based on fusions of unique promoter fragments and firefly luciferase.

Four different rat NHE3 gene promoter constructs (pGL3/−1360 bp, pGL3/−548 bp, pGL3/−149 bp, and pGL3/−95 bp) transfected into OKP cells, demonstrated significant activity. Furthermore, the two longer promoter constructs (pGL3/−1360 bp and pGL3/−548 bp) were responsive to A-II treatment, whereas two shorter 5′-delection constructs (pGL3/−149 bp and pGL3/−95 bp) were not. Interestingly, promoter construct pGL3/−1360 bp showed a ∼44% increase by A-II treatment, whereas the pGL3/−548 bp promoter construct showed a ∼74% increase by A-II treatment. These observations suggest that the putative A-II responsive element(s) is located between 149 and 548 bp upstream of the transcription initiation site, with a modulatory *cis*-element(s) located further upstream, between −548 and −1360 nt.

A-II is known to exert its effects through two different A-II receptor subtypes, designated as type 1 receptor (AT1-R) and type 2 receptor (AT2-R) [35]. Many known biological actions of A-II are mediated by stimulation of the AT1-R, which is a member of the G protein-coupled seven-transmembrane-spanning receptor family [36]. Cano et al. demonstrated that acute treatment with A-II at physiological concentrations stimulates NHE activity in OKP cells via a cAMP-independent mechanism mediated by AT1-R and a pertussis toxin-sensitive G protein [20]. Therefore, it is possible that the effect of A-II on NHE3 gene expression in OKP cells is mediated by AT1-R in our experiments. Several A-II responsive elements mediated by AT1-R signal transduction have been identified within the interleukin-6 [35], the plasminogen-activator inhibitor type-1 (PAI-1) [37], and the cyclin D1 genes [26]. These A-II responsive elements include a cAMP response element (CRE)-binding sequence in the interleukin-6 gene [35], an Sp1 binding sequence in the PAI-1 gene [37], and an early growth response gene-1 (Egr-1) binding sequence in the cyclin D1 gene [26]. We searched the rat NHE3 gene promoter region (−548 bp to −149 bp), and found that one sequence (−395 bp-CGGGGGCGGTGCA-382 bp) is identical to the Sp1 binding sequence which appears in the PAI-1 gene [37]. It remains to be determined whether binding of Sp1 transcription factor to this putative *cis*-element participates in the mechanism of A-II regulation of the rat NHE3 gene transcription.

In summary, we demonstrate that prolonged exposure of renal proximal tubule epithelial cells to A-II in a concentration previously demonstrated to acutely inhibit Na^+/H^+ exchange [11,12] results in transcriptionally mediated increase in NHE3. This finding adds a new dimension to the biphasic and concentration-dependent effects of angiotensin II on NHE3-mediated renal acidification, and epithelial Na^+ and HCO_3^- reabsorption. Further studies focusing on identification of the A-II responsive element(s) and *trans*-factors involved in A-II regulation of the NHE3 gene are required to fully define the transcriptional mechanism underlying this phenomenon.

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