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Cardiac glycoside downregulates NHE3 activity and expression in LLC-PK₁ cells

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Oweis, Shadi, Liang Wu, Pawel R. Kiela, Hui Zhao, Deepak Malhotra, Fayez K. Ghishan, Zijian Xie, Joseph I. Shapiro, and Jiang Liu. Cardiac glycoside downregulates NHE3 activity and expression in LLC-PK₁ cells. *Am J Physiol Renal Physiol* 290: F997–F1009, 2006. First published December 13, 2005; doi:10.1152/ajprenal.00322.2005.—Ouabain, a cardiotonic steroid and a specific inhibitor of the Na⁺-K⁺-ATPase, has been shown to significantly inhibit transcellular Na⁺ transport without altering the intracellular Na⁺ concentration ([Na⁺]_i) in the epithelial cells derived from the renal proximal tubules. We therefore studied whether ouabain affects the activity and expression of Na⁺/H⁺ exchanger isoform 3 (NHE3) representing the major route of apical Na⁺ reabsorption in LLC-PK₁ cells. Chronic basolateral, but not apical, exposure to low-concentration ouabain (50 and 100 nM) did not change [Na⁺]_i but significantly reduced NHE3 activity, NHE3 protein, and mRNA expression. Inhibition of c-Src or phosphoinositide 3-kinase (PI3K) with PP2 or wortmannin, respectively, abolished ouabain-induced downregulation of NHE3 activity and mRNA expression. In caveolin-1 knockdown LLC-PK₁ cells, ouabain failed to downregulate NHE3 mRNA expression and NHE3 promoter activity. Ouabain response elements were mapped to a region between -450 and -1,194 nt, where decreased binding of thyroid hormone receptor (TR) and Sp1 to their cognate *cis*-elements was documented *in vitro* and *in vivo* by protein/DNA array analysis, EMSA, supershift, and chromatin immunoprecipitation. These data suggest that, in LLC-PK₁ cells, ouabain-induced signaling through the Na⁺-K⁺-ATPase-Src pathway results in decreased Sp1 and TR DNA binding activity and consequently in decreased expression and activity of NHE3. These novel findings may represent the underlying mechanism of cardiotonic steroid-mediated renal compensatory response to volume expansion and/or hypertension.

kidney; sodium; Slc9a3; caveolin-1; c-Src

ENDOGENOUS CARDIOTONIC STEROIDS (also referred to as endogenous digitalis-like substances or DLS), including ouabain and marinobufagenin (MBG), are now accepted as a class of steroid hormones involved in blood pressure regulation and renal sodium handling (44, 53). Elevated endogenous ouabain levels have been found under a number of conditions such as sodium imbalance, chronic renal failure, hyperaldosteronism, hypertension, congestive heart failure, and preeclampsia (21, 23–25). Elevated plasma MBG concentrations have been associated with volume expansion and hypertension (17). The increased circulating endogenous digitalis-like compound(s) contribute to hypertension through inhibition of Na⁺-K⁺-ATPase and Ca²⁺-mediated positive inotropic effects in cardiovascular tissues, whereas in renal epithelium, the effects of DLS may represent an adaptive response, where inhibition of the sodium pump would induce excess sodium excretion in the

proximal tubule and consequently a reduction of the extracellular fluid (ECF) volume (24). This mechanism is in agreement with an early concept of an endogenous natriuretic compound introduced by Dahl (13), deWardener (14), and Blaustein (5).

In proximal tubule cell, the Na⁺-K⁺-ATPase resides at the basolateral surface, providing the driving force for the vectorial transport of sodium from the tubular lumen to the vascular compartment (9). We previously reported that in LLC-PK₁ cells, low-concentration ouabain induces significant depletion of the basolateral Na⁺-K⁺-ATPase and decreases in transcellular ²²Na⁺ transport without intracellular Na⁺ changing (34, 36). Novel findings indicate that in addition to exchanging ions, Na⁺-K⁺-ATPase also functions as a signal transducer, leading to the activation of a signaling cascade involving c-Src and epidermal growth factor receptor (EGFR) (22, 37). Ouabain induces endocytosis of the Na⁺-K⁺-ATPase α₁-subunit, a process mediated by c-Src and PI3K kinases (34), and caveolin-1 (35). Moreover, in male Sprague-Dawley rats, high-salt diet (4% NaCl) increased urinary sodium and MBG excretion, reduced proximal tubular Na⁺-K⁺-ATPase enzymatic activity, and induced endocytosis of proximal tubular Na⁺-K⁺-ATPase α₁-subunit. These effects were significantly attenuated by administration of an anti-MBG antibody (42), suggesting that redistribution of Na⁺-K⁺-ATPase in the epithelium of the renal proximal tubules in response to endogenous cardiac steroids plays an important role in renal adaptation to salt loading.

Collectively, these data support the notion that DLS might cause a physiologically relevant reduction in transcellular sodium transport in the renal proximal tubule. For this mechanism to function in the physiological setting, however, the apical component of proximal tubule Na⁺ reabsorption would also have to be negatively affected by cardiac glycosides. This apical component is partially represented by NHE3, a plasmalemmal sodium-hydrogen exchanger expressed in the apical membrane in the proximal tubule and in the cortical thick ascending limb (TAL) of the loop of Henle (2, 4). NHE3 has been unequivocally demonstrated to provide a critical mechanism for Na⁺, HCO₃⁻, and fluid reabsorption in the proximal tubule (45). Therefore, we hypothesized that the basolaterally localized Na⁺-K⁺-ATPase and apically localized NHE3 work in concert to regulate renal sodium handling in response to DLS. NHE3 has been shown to be redistributed under hypertensive state (56), accompanying reversible downregulation of the Na⁺-K⁺-ATPase activity in the renal cortex (59, 60).

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NHE3 activity is regulated at various levels including phosphorylation which requires ezrin and NHERFs (61), trafficking of NHE3 protein on and off the apical membrane (57), association with lipid rafts in the brush-border membrane (32), and by transcriptional regulation of NHE3 mRNA level. The latter mechanism of regulation has been characterized best for rat (6, 27, 30) and to lesser extent human (38) gene promoters. Sequence analysis of the rat NHE3 gene promoter revealed the presence of numerous putative *cis*-acting elements recognized by various transcription factors (e.g., AP-1, AP-2, C-EBP, NK-1, OCT-1/OTF-1, Sp1, glucocorticoid, and PTH receptors) (6, 27). Rat NHE3 promoter has been described as initiator-driven and controlled mainly by Sp1 and Sp3, which functionally interact with GATA-5 (30). Physiological modulators of NHE3 gene transcription include glucocorticoid hormones (6, 28), thyroid hormone (7), protein kinase C (1), and sodium butyrate (29).

Although the effect of cardiotonic steroids on NHE3-mediated sodium handling in the kidney remains unknown, we hypothesized that a mechanism involving this crucial renal apical transport protein complements changes in basolateral Na⁺ transport in response to ouabain. To test ouabain's effects on NHE3 in the proximal tubule, we investigated the molecular mechanism of long-term, low-concentration ouabain on NHE3 activity and expression. Our results demonstrated that ouabain decreases apical NHE3-mediated Na⁺ absorption, NHE3 protein, and mRNA abundance in LLC-PK₁ cells. Our data also suggested that ouabain-induced signaling through the Na⁺-K⁺-ATPase-Src might be essential for ouabain-induced down-regulation of NHE3, in a mechanism mediated at least partially, through Sp1 transcription factor.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma (St. Louis, MO). All PCR reagents were purchased from Promega (Madison, WI). PP2 and wortmannin were obtained from Calbiochem (San Diego, CA). ²²Na⁺ was obtained from DuPont New England Nuclear Life Science (Boston, MA).

A rabbit polyclonal antibody against a mixture of peptides of pig NHE3 (residue 26: C-AQGTEEVPGDAHGD; residue 758: C-PVFSPEDEPDSILSR; and residue 819: C-EEQPRAAPAESTHM) was prepared and affinity purified. This antibody specifically recognized a protein band ~90 kDa. Polyclonal antibody raised against a fusion protein containing 699–831 aa from COOH terminus of rat NHE3 produced by baculovirus expression in Sf9 insect cells (12) was used to detect NHE3 protein in rat cardiac fibroblasts. Antibody against Sp1 (for ChIP assays) and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against Sp1 (for supershift assays) was obtained from Geneka (Montreal, Quebec).

Cell culture. LLC-PK₁ cell line was obtained from ATCC (Manassas, VA). P-11 (control) and C2-9 (siRNA-mediated caveolin-1 knockdown) cell lines were generated and cultured as described earlier (36, 52). All cells were serum-starved for 12 h before treatment. Adult cardiac fibroblasts were prepared from the ventricles of one or two adult male Sprague-Dawley rats. Calcium-tolerant adult cardiac myocytes were prepared and pelleted as described (48), the supernatant containing fibroblasts was further pelleted (1,000 rpm for 10 min) and resuspended in DMEM supplemented with streptomycin, penicillin, fungizone, and 10% FBS. Fibroblasts were allowed to attach to tissue culture plates for 30 min. Unattached cells were rinsed free and discarded. Cell passage 2-3 was used in all the experiments. The purity (>95%) of the fibroblasts was determined by the expressions of vimentin and collagen (type I and type III), as described (39). Expression of NHE3 was determined by immunoblotting of membrane protein fraction with anti-rat-NHE3 antibody.

Intracellular sodium ([Na⁺]_i) measurements. [Na⁺]_i was measured as previously described (41). After treatment, LLC-PK₁ cells or primary cardiac fibroblasts were loaded with 10 μM sodium-sensitive fluorescence dye SBFI-AM (Molecular Probes, Eugene, OR) in the presence of 0.2% pluronic F-127 (Molecular Probes) in HBS buffer (in mM: 140 NaCl, 4.6 KCl, 10 HEPES, 10 glucose, 2 CaCl₂, 1 MgCl₂, pH 7.4 adjusted with Tris). Fluorescence emission was monitored at 480 nm to obtain excitation ratios (F340/F380), using a computer-controlled spectrophotometer (PTI, London, Canada) with Felix software (PTI). Calibration was performed as described elsewhere ($R^2 = 0.98$, $n = 6$) (15).

Quantitative measurement of NHE3 mRNA. Total RNA was isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH), according to manufacturer's protocol and mRNA and cDNA were prepared as described (54). Standardized reverse-transcriptase PCR (StaRT-PCR) (54), a modification of the competitive template (CT) RT method described by Gilliland et al. (19), was used to measure gene expression, with GAPDH transcript used as an internal control. cDNA, CT mixture, and standardized mixture of internal control were prepared as described (54). Target genes (NHE3 and GAPDH) were amplified in the presence of specific primers and CTs (see Table 1 for sequences) for 35 cycles in Rapidcycler air thermocycler (Idaho technology, Salt Lake, UT). PCR reactions volume was 10 μl containing 50 ng of each primer, 1 μl 10× buffer, 0.5 U of *Taq* polymerase, 0.2 mM dNTPs, and 1 μl CT mix and cDNA. Each cycle included denaturation 5 s at 94°C, annealing 10 s at 58°C, and then elongation 15 s at 72°C with a slope of 9.9.

NHE3 activity measurements. Intracellular pH and ²²Na⁺ uptake were determined as described (46). Calibration of intracellular pH was performed using high K⁺-nigericin (10 μM) standards as described ($R^2 = 0.99$, $n = 9$) (40). During measurement of intracellular pH and ²²Na⁺ uptake, 50 μM amiloride was used to inhibit amiloride-sensitive NHE1 activity. Transcellular ²²Na⁺ transport assay was performed in LLC-PK₁ monolayers (grown on Transwell membrane support) as described previously (36). LLC-PK₁ monolayers were treated with ouabain (100 nM, 12 h) applied in the basolateral or the apical compartment. Transcellular (apical to basolateral) ²²Na⁺ flux

Table 1. Primers used in StaRT-PCR for quantitative gene expression assays

Primer	Sequence (5' to 3')	Position	Size, bp	GenBank
GAPDH				
Forward(F)	gAC CCC TTC ATT gAC CTC AA	163–182		BC013310.2
Reverse(R)	TgC TTC ACC ACC TTC TTg AT	851–832	689	
CT	R+gAT gAC CTT gCC CAC AgC CT	R+723–704	581	
NHE3				
Forward(F)	GAA GTA CGT GAA GGC CAA CA	1–20		AF123280
Reverse(R)	GAC GAT GAC GGT GAA GAA GA	394–375	393	
CT	R + CTC CTT GAC CTT GTT CTC GT	R+340–321	360	

was determined by counting radioactivity in the basolateral aspect at indicated time points.

NHE3 protein measurements. Whole cell lysate was prepared using modified RIPA buffer, as described earlier (36). Crude membrane fraction was isolated as described by Bacic et al. (3). Equal amounts of total protein were resolved by 10% SDS-PAGE and immunoblotted with a polyclonal antibody against NHE3. The same membrane was stripped and immunoblotted with anti-tubulin antibody to serve as an internal loading control. The densities of NHE3 and tubulin bands were quantified using Molecular Analyst software (Bio-Rad, Hercules, CA).

Labeling of cell-surface proteins by biotinylation. Cell-surface protein biotinylation was performed as described elsewhere (20, 34). Proteins bound to the ImmunoPure immobilized streptavidin-agarose beads were eluted and then resolved by SDS-PAGE followed by immunoblotting.

Measurement of the NHE3 promoter activity. NHE3 promoter constructs (in pGL-3 basic luciferase reporter vector, Promega) were previously described (30). The luciferase-reporter plasmid, Sp1-luc, which contains the sequence of 5'-CGCGTGGGCGGA-CTGGGCGGAGTTAGGGGCGGGA-3', consisting of three consensus Sp1 binding sites (GGGCGG) from the SV40 promoter, was generated by subcloning the above fragment into *Sma*I site in pGL3-basic, according to Sowa et al. (47). LLC-PK₁ cells were grown to 70–80% confluence in 24-well plates and transiently transfected with selected constructs using FuGENE6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. After transfection and desired treatments, cells were washed three times with ice-cold PBS and lysed with passive lysis buffer (PLB) (Promega). The firefly luciferase activity was measured by Lumat LB 9507 tube luminometer (Berthold Technologies) using Luciferase Assay System (Promega). The obtained data were normalized to protein concentration.

TranSignal protein/DNA array I. TranSignal Protein/DNA Array (preparation of transcriptional factor-bound DNA, hybridization, detection, and analysis) was performed according to manufacturer's protocol (Panomics, Redwood City, CA).

Preparation of nuclear extracts and EMSA. Nuclear extracts were prepared as described (16). Protein concentration was determined by the Bradford method (Bio-Rad) and samples were stored at -70°C until use. For EMSA, double-strand oligonucleotides (IDT, Coralville, IA) were end-labeled with [γ - ^{32}P]ATP by T4 polynucleotide kinase (Promega). Assays were performed by incubating 5 μg of nuclear protein with 5 pmol of labeled probe for 20 min. Binding reactions were carried out at room temperature for 20 min in binding buffer (50 mM Tris·HCl, pH 7.5, 5 mM MgCl₂, 2.5 mM EDTA, 20% glycerol, 2.5 mM dithiothreitol, and 250 mM NaCl). Excess of competing unlabeled oligonucleotides was added 20 min before addition of the labeled probe. When using supershift to identify protein in the protein-DNA complexes, nuclear extracts were incubated with 2.5 μg of the appropriate antibody at room temperature for 30 min followed by the addition of labeled probe and a further incubation at room temperature for 20 min. The reaction mixtures were resolved on 4% polyacrylamide gels and subjected to autoradiography.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation, or ChIP, refers to a procedure used to determine whether a given protein binds to or is localized to a specific DNA sequence in vivo. In this assay, DNA-binding proteins are cross-linked to DNA with formaldehyde in living cells. Chromatin is then isolated and sheared along with bound proteins into small fragments and used as a substrate for immunoprecipitation with antibodies specific to the DNA-binding protein. After reversing the cross-linking to release the

DNA, PCR is used to amplify specific DNA sequences to see whether they were precipitated with the antibody. The protocol for this assay was adapted from ChIP Assay Kit (Upstate Biotechnology, Waltham, MA). LLC-PK₁ cells or adult rat cardiac fibroblasts were fixed with formaldehyde (1%) and harvested with ice-cold PBS. The following procedures were performed at 4°C unless stated otherwise. Cell pellets were suspended in 10 ml ChIP lysis buffer and incubated for 10 min. The nuclear pellets were collected after centrifugation at 600 *g* for 5 min and resuspended in 10 ml washing buffer and incubated for 10 min. The nuclei were collected by centrifugation and resuspended in 2 ml radioimmunoprecipitation assay (RIPA) buffer. Samples were sonicated three times on ice with a sonic dismembrator (Fisher Scientific) at output 4 for 20 s followed by 50-s pulse-off time. Samples were then centrifuged twice at 16,000 *g* for 10 min. Aliquoted cleared chromatin extracts were then diluted to 1 ml with ChIP dilution buffer. Ten micrograms of anti-Sp1 or 10 μg normal rabbit IgG (Santa Cruz Biotechnology) were then added and the reaction mixtures were incubated overnight on a rotary shaker. The samples were then mixed with sonicated salmon sperm DNA (100 $\mu\text{g}/\text{ml}$) and 50 μl of 50% protein A Sepharose beads and incubated for 3 h. After centrifugation (16,000 *g* for 10 s), the pellets were washed (with rotation) once with RIPA buffer for 5 min; once with RIPA buffer containing 500 mM NaCl for 5 min; once with LiCl buffer for 10 min; four times with TE buffer and once with RIPA buffer for 5 min. The samples were suspended in 100 μl digestion buffer and incubated at 55°C for 3 h and then at 65°C for 6 h. The samples were treated with 10 $\mu\text{g}/\text{ml}$ RNAase A at 37°C for 1 h, extracted once with phenol-chloroform and once with chloroform, and precipitated in the presence of 0.3 M sodium acetate/0.01 M MgCl₂ in 5 Vol of ethanol at -80°C for 30 min. The DNA pellets were dissolved in 50 μl of elution buffer (10 mM Tris·HCl, pH 8.5). PCR amplification of a 186-bp fragment of the NHE3 promoter containing an Sp consensus element was carried out using primer pair presented in Table 2. Another upstream primer pair was used to amplify a 210-bp fragment ($-1,365$ nt to $-1,155$ nt; Table 2) serving as a negative control.

Statistical analysis. Data were first tested for normality (all data passed) and then subjected to parametric analysis. When more than two groups were compared, one-way ANOVA was performed before comparison of individual groups with the unpaired Student's *t*-test with Bonferroni's correction for multiple comparisons. If only two groups of normal data were compared, the Student's *t*-test was used without correction (51). SPSS software was used for all analysis (SPSS, Chicago, IL).

RESULTS

Low-concentration ouabain does not alter intracellular sodium concentration in LLC-PK₁ cells or cardiac fibroblasts. Ouabain significantly reduced transcellular Na⁺ transport in LLC-PK₁ monolayers (36) and activated signaling pathways that are independent of [Na⁺]_i change in cardiac myocytes (37). In LLC-PK₁ cells, ouabain treatment (100 nM for 12 h) did not change [Na⁺]_i (14.4 ± 1.4 and 13.96 ± 2.02 mM in treated and control groups, respectively, $n = 6$). However, in cells treated with 1 mM ouabain, [Na⁺]_i was still significantly increased within 5 min ($[\text{Na}^+]_i = 21.5 \pm 2.5$ mM, $n = 6$), consistently with previous observations that high concentration of ouabain is required to alter intracellular Na⁺ (41). To evaluate primary rat cardiac fibroblasts as a rat cell model for downstream studies, isolated cells were

Table 2. PCR primers for ChIP assay

(-604 to -418 nt)	ATTCTTGACTCTCACTTGGCGGGA	CCCGTTATATACACTCAGCAAGGC
(-1,365 to -1,155nt)	TAGGTCGTGAGCATGGAGAGGT	ACCACTTAAGCCTTGAAACCC

treated with 10 or 25 μM ouabain for 12 h. Higher ouabain concentration utilized in studies with cardiac fibroblasts was dictated by the predominant expression of a relatively ouabain-resistant isoform of $\text{Na}^+\text{-K}^+\text{-ATPase}$ α_1 -subunit in rodents. Neither concentration of ouabain has a significant effect on $[\text{Na}^+]_i$ (22.1 ± 2.4 , 21.7 ± 2.3 , and 22.7 ± 2.6 mM in fibroblasts treated with no ouabain, 10 μM , or 25 μM ouabain, respectively, $n = 4$).

Ouabain significantly inhibits NHE3 activity in LLC-PK₁ cells. Because ouabain significantly reduced transcellular Na^+ transport without inducing $[\text{Na}^+]_i$ change, we assessed the effect of low-concentration ouabain on NHE3 activity, by measuring the Na^+ -dependent intracellular pH recovery and H^+ -driven $^{22}\text{Na}^+$ uptake. As shown in Fig. 1A, ouabain treatment significantly decreased H^+ -driven $^{22}\text{Na}^+$ uptake in a time-dependent manner. To further confirm that ouabain-in-

duced decrease in $^{22}\text{Na}^+$ uptake is due to the inhibition of NHE3 activity, we also measured Na^+ -stimulated pH recovery rate after Na^+ reintroduction. After ouabain treatment (100 nM, 12 h), Na^+ -stimulated pH recovery rate was also decreased by $\sim 50\%$ (0.15 ± 0.04 pH U/min, $n = 12$) compared with control cells (0.3 ± 0.05 pH U/min, $n = 9$; $P < 0.01$; Fig. 1B). To evaluate ouabain effect on transcellular Na^+ transport, LLC-PK₁ cells were grown on Transwell membrane support to form a monolayer. Ouabain (100 nM, 12 h) was applied to the basolateral or apical aspect, and transcellular $^{22}\text{Na}^+$ flux from apical to basolateral compartments was determined as described earlier (36). Ouabain treatment in the basolateral aspect significantly reduced transcellular $^{22}\text{Na}^+$ flux, but no such effect was observed with apical exposure of LLC-PK₁ monolayer to ouabain ($n = 4$; Fig. 1C). These data suggest that ouabain-induced inhibition of NHE3 activity is due to its

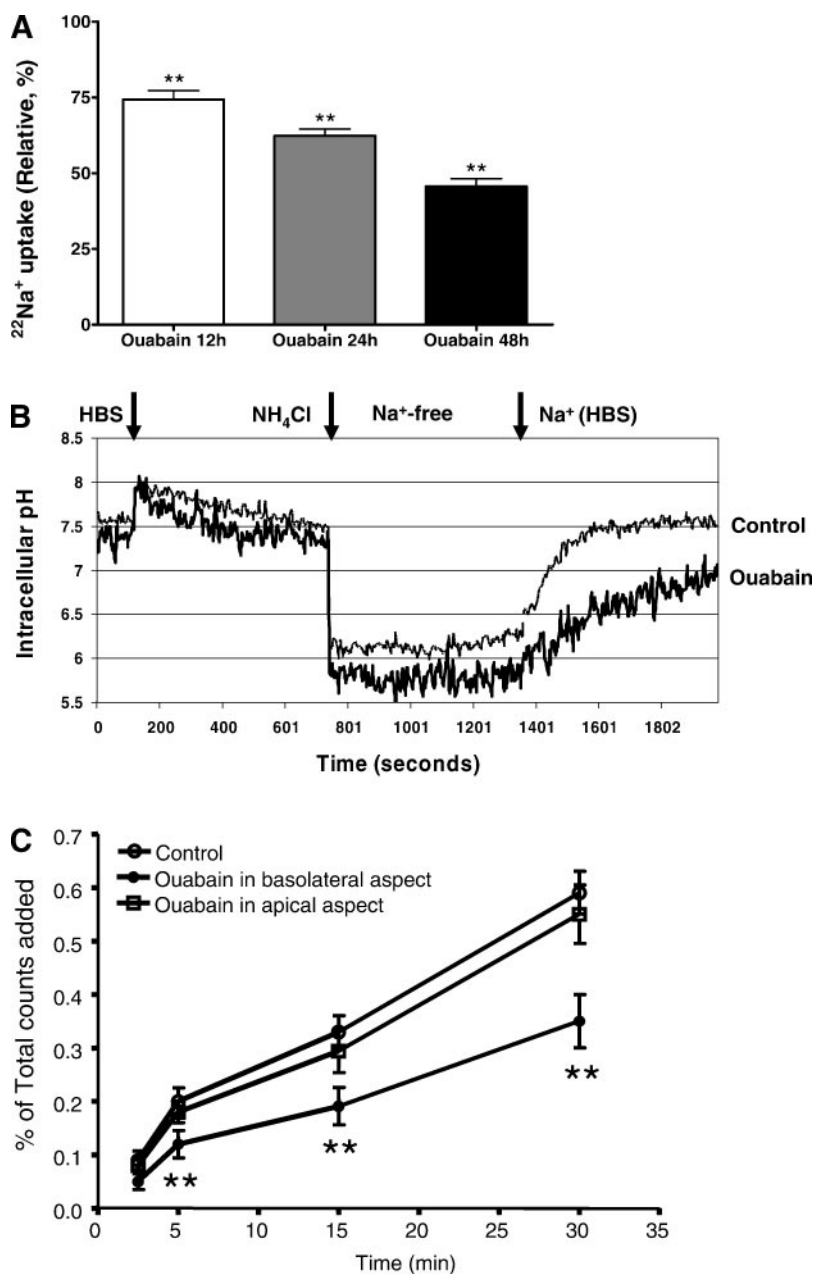


Fig. 1. Ouabain treatment decreases the Na^+ -dependent intracellular pH recovery and H^+ -driven $^{22}\text{Na}^+$ uptake. LLC-PK₁ cells were treated without or with 100 nM ouabain for 12–48 h. Na^+ -dependent intracellular pH recovery and H^+ -driven $^{22}\text{Na}^+$ uptake were measured as described in MATERIALS AND METHODS. A: H^+ -driven $^{22}\text{Na}^+$ uptake after ouabain (100 nM). Values are means \pm SE. $**P < 0.01$ ouabain vs. control, $n = 6$. B: representative pH recovery rate after 12-h ouabain treatment (100 nM). C: effects of ouabain (100 nM, 12 h) applied in the basolateral or apical aspect of LLC-PK₁ monolayers grown on Transwell filter support. Values are means \pm SE. $**P < 0.01$ ouabain vs. control or ouabain apical vs. ouabain basolateral, $n = 4$.

effect(s) on the Na⁺-K⁺-ATPase, which is exclusively expressed on the basolateral membrane.

Ouabain downregulates NHE3 protein and mRNA abundance. As demonstrated above, low-concentration ouabain inhibited NHE3 activity and Na-K-ATPase activity (36) with-

out a concomitant change in [Na⁺]_i. To determine the potential effects of ouabain on NHE3 expression, we utilized Western blotting and quantitative RT-PCR to determine NHE3 protein and mRNA expression in LLC-PK₁. As shown in Fig. 2, ouabain treatment induced a significant and time-dependent decrease in NHE3 protein content in whole cell lysate ($P < 0.01$, $n = 6$; Fig. 2A), as well as in the crude membrane fraction ($P < 0.01$, $n = 4$; Fig. 2B). No NHE3 was detected in the cytosol (up to 60 μg of total proteins, data not shown). Moreover, cell-surface biotinylation experiment also showed a significant decrease in plasmalemmal NHE3 protein content in response to 100 nM ouabain added into the basolateral ($P < 0.01$, $n = 3$; Fig. 2C), but not into the apical compartment (data not shown). Ouabain had similar effects on NHE3 expression in primary rat cardiac fibroblasts. Western blot analysis of crude membranes obtained from these cells utilizing a rat NHE3-specific antiserum demonstrated easily detectable expression of NHE3 protein, which was dose dependently down-regulated with ouabain (Fig. 2D). Next, we investigated the effect of ouabain on NHE3 mRNA expression by StaRT-PCR as a quantitative gene expression assay with GADPH as an internal control. These studies yielded results which highly correlated with those observed for NHE3 protein expression. After 24-h ouabain treatment at 50 or 100 nM, we found that in LLC-PK₁ cells, NHE3 mRNA expression was decreased by ~40 and 55%, respectively, compared with control ($P < 0.01$, $n = 6$; Fig. 3A). Ouabain (100 nM)-induced downregulation of NHE3 mRNA was also shown to be time dependent (Fig. 3B).

Ouabain inhibits NHE3 gene promoter activity in LLC-PK₁ cells. Because ouabain downregulated NHE3 protein and mRNA expression, it was of interest to explore the possible effect of ouabain on NHE3 gene promoter regulation. Because pig NHE3 gene promoter has not been cloned, we chose well-described and -characterized rat NHE3 gene promoter for mechanistic studies related to NHE3 gene transcription. LLC-PK₁ were transiently transfected with the indicated reporter plasmids and treated without (as control) or with 100 nM ouabain for 24 h. As depicted in Fig. 4, ouabain treatment significantly inhibited luciferase activity driven by the -1,360/+58-bp fragment of the rat NHE3 gene promoter ($n = 3$, $P < 0.01$). To provide initial mapping of the putative *cis*-elements and to facilitate identification of transcription factor(s) involved in the response to ouabain, we then used various reporter constructs containing progressive 5'-deletions of the

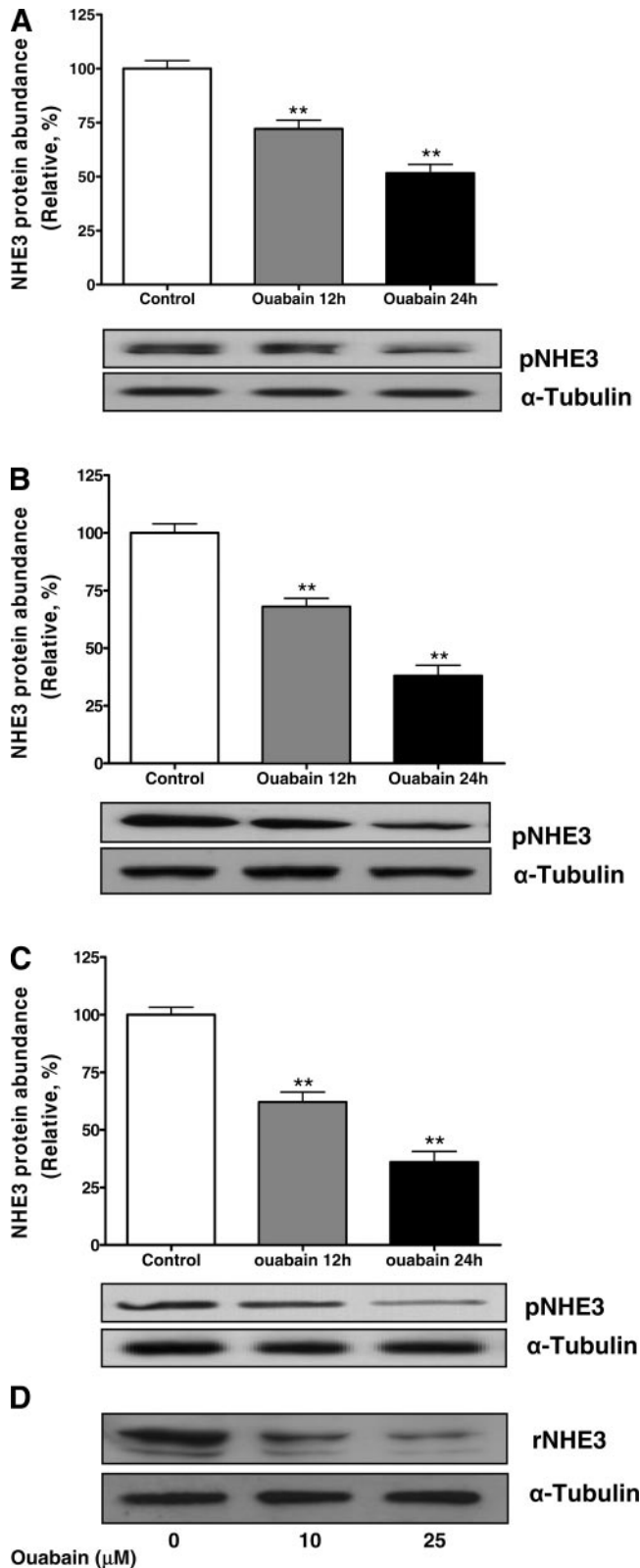


Fig. 2. Ouabain treatment decreases NHE3 protein abundance in LLC-PK₁ cells and in primary adult cardiac fibroblasts. LLC-PK₁ cells were treated without or with 100 nM ouabain for 12 or 24 h. NHE3 protein and mRNA abundance were determined as described in MATERIALS AND METHODS. Primary adult rat cardiac fibroblasts were treated without or with 10 or 25 μM ouabain for 12 h. A: representative Western blot of whole cell lysate after ouabain treatment and a bar graph summary of immunoblotting data. Values are means ± SE expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 5$. B: representative Western blot of crude membrane-associated NHE3 protein abundance after ouabain treatment and a bar graph summary of immunoblotting data. Values are means ± SE expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 4$. C: representative Western blot of plasmalemmal NHE3 as detected by cell-surface protein biotinylation after ouabain treatment and a bar graph summary of immunoblotting data. Values are means ± SE expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 3$. D: representative Western blot of crude membrane-associated NHE3 protein abundance in control and ouabain-treated adult rat cardiac fibroblasts.

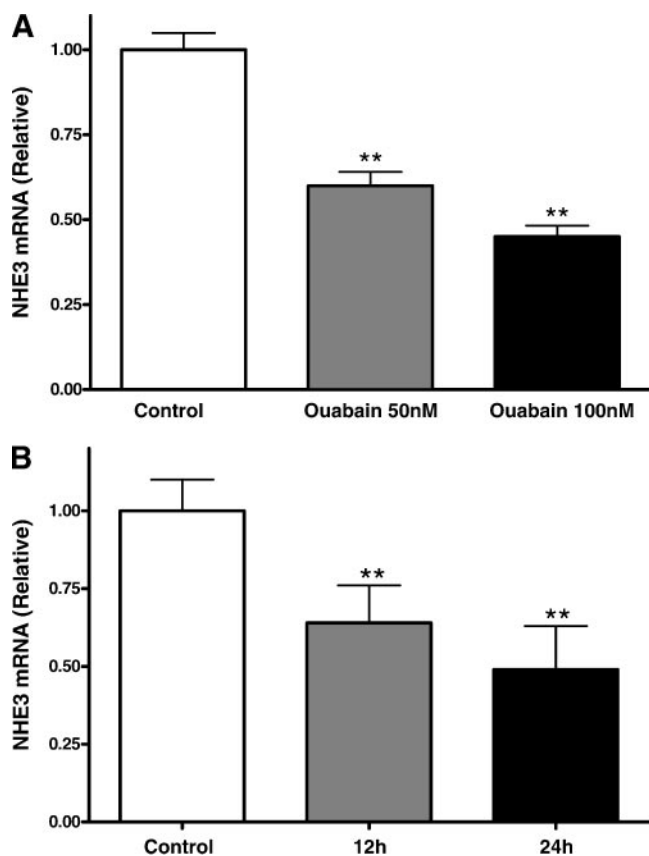


Fig. 3. Ouabain treatment decreases NHE3 mRNA abundance in LLC-PK₁ cells. **A**: concentration-dependent effect of ouabain (24 h) on NHE3 mRNA expression as quantified by StarT-PCR. The expression of GAPDH was used as an internal control. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 6$. **B**: time-dependent effect of ouabain (100 nM) on NHE3 mRNA expression as quantified by StarT-PCR. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 6$.

NHE3 promoter (29). As illustrated in Fig. 5, ouabain-mediated downregulation of NHE3 promoter activity involves two or more *cis*-elements located between $-1,194/-715$ and $-715/-450$ nt.

c-Src, *PI3K*, and *caveolin-1* are involved in ouabain's effect on NHE3 regulation. Because previous studies implicated *c-Src* and *PI3K* kinases as critical components of ouabain-triggered signaling cascade, we tested whether these two kinases are involved in ouabain-induced downregulation of NHE3. LLC-PK₁ cells were preincubated with specific inhibitor of *c-Src* or *PI3K* (1 μ M PP2 or 100 nM wortmannin, respectively) for 30 min and then treated with ouabain (100 nM, 24 h) in the presence of a respective inhibitor. As shown in Fig. 6A, inhibition of *c-Src* or *PI3K* by preincubation with 1 μ M PP2 or 100 nM wortmannin abolished ouabain-induced downregulation of NHE3 mRNA, whereas neither inhibitor alone affected NHE3 expression. To further confirm this observation at a functional level, we also determined the effect of PP2 and wortmannin on H⁺-driven ²²Na⁺ uptake. As shown in Fig. 6B, inhibition of *c-Src* or *PI3K* abolished ouabain-induced downregulation of NHE3 activity, whereas PP2 or wortmannin alone was without effect. These observations strongly suggest that *c-Src* and *PI3K* kinase activities are involved in the

mechanism of ouabain action on NHE3 activity and expression.

We next explored the possible involvement of caveolin-1 which has been described as indispensable for ouabain-induced signal transduction, endocytosis of the Na⁺-K⁺-ATPase α_1 -subunit, and compartmentalization of signaling molecules (35, 52). To address this hypothesis, we utilized previously described (36, 52) LLC-PK₁ cells stably transfected with an empty vector (P-11, as control) or a vector expressing caveolin-1-specific siRNA (C2-9, as caveolin-1 knockdown cells). As shown in Fig. 7, ouabain treatment (100 nM, 24 h) inhibited NHE3 mRNA expression in P-11 cells similarly to the effect seen in the wild-type LLC-PK₁ cells, but not in the caveolin-1 knockdown C2-9 cells. As depicted in Fig. 8, ouabain also inhibited NHE3 promoter activity ($-1,194/+58$) in transiently transfected P-11 cells, but not in the caveolin-1-deficient C2-9 cells, indicating that ouabain-induced reduction of NHE3 gene transcription requires caveolin-1.

Transcriptional mechanism of ouabain-mediated regulation of NHE3 gene. To identify transcription factor(s) with DNA binding affinities modified by ouabain treatment, we used TranSignal Array (TranSignal Protein/DNA Spin Array I, Panomics) to screen the formation of transcription factor/DNA complexes, followed by EMSA to verify the obtained results and confirm the identity of putative *trans*-factors interacting with the putative elements in the NHE3 promoter. For these studies, we utilized control or ouabain-treated (100 nM, 12 or 24 h) LLC-PK₁ cells or primary cardiac fibroblasts as a model rat cell line. The analysis of the obtained TranSignal Array data identified significant and time-dependent decrease in binding of thyroid hormone receptor (TR) and Sp1 transcription factors to their respective *cis*-elements in both rat cardiac fibroblasts and in LLC-PK₁ cells (data not shown).

We further aimed to examine the association of nuclear proteins implicated by the TranSignal Arrays with putative *cis*-elements located within NHE3 promoter regions indicated by studies with deletion constructs ($-1,194/-715$ and $-715/-450$ nt). Based on prediction analysis, we designed and synthesized the following double-strand DNA probes: $-1,024$ -TRE (TR element) with three TR binding sites (27) and

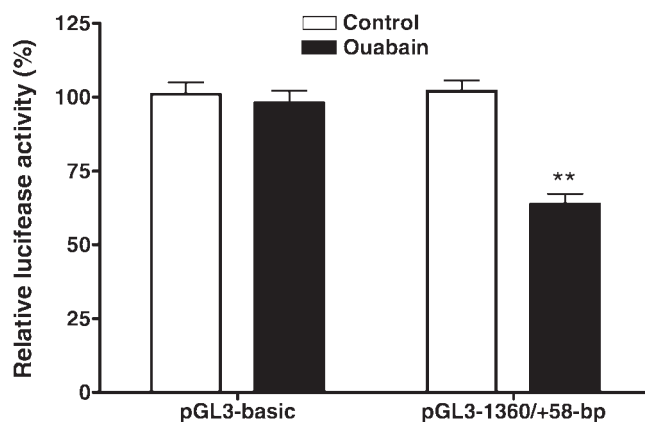


Fig. 4. Ouabain inhibited the activity of -1.4 kb of NHE3 promoter transiently transfected LLC-PK₁ cells. Cells transfected cells with promoterless pGL3-basic or with pGL3- $1,360/+58$ -nt construct were treated with control or with 100 nM ouabain-supplemented medium for 24 h. Luciferase activity was normalized to protein concentration. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 3$.

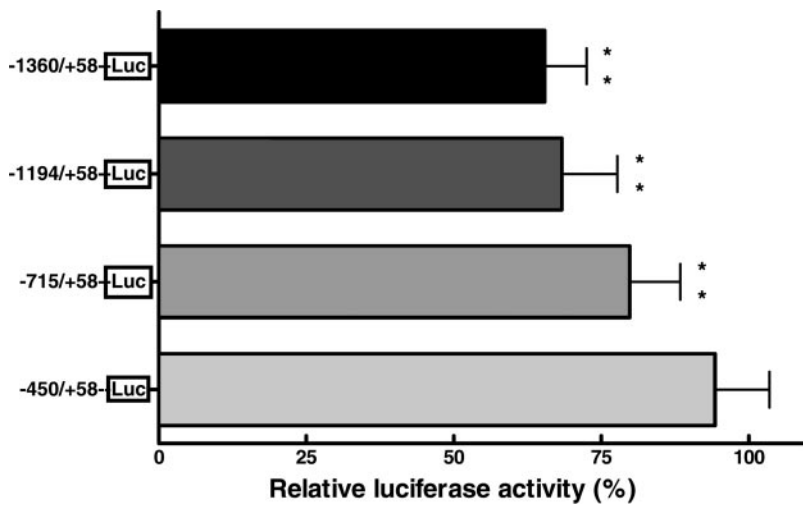


Fig. 5. Deletion analysis of ouabain-induced downregulation of NHE3 promoter activity. LLC-PK₁ cells were transfected with progressively truncated NHE3 promoter constructs and treated with ouabain (100 nM) for 24 h. Luciferase activity was normalized to protein concentration. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 6$.

–592-Sp1 probe with one Sp1 binding site (27) (see Table 3). As controls we used TRE and Sp1 consensus oligonucleotides: GAT CGT AAG ATT CAG GTC ATG ACC TGA GGA GA and ATT CGA TCG GGG CCG GGC GAG, respectively. In

rat cardiac fibroblasts, ouabain reduced the formation of protein-DNA complexes with TRE and Sp1 probes, suggesting the involvement of these two *cis*-elements in the molecular mechanism of ouabain-induced regulation of NHE3 gene transcription (Fig. 9). More importantly, our data also showed that heterologous TRE or Sp1 consensus oligonucleotides completely blocked binding to ³²P-labeled NHE3 promoter-derived TRE or Sp1 probe, respectively. These observations were confirmed in EMSA analysis of nuclear protein from LLC-PK₁ cells (data not shown). To verify the results of the gel shift assay and to further identify the binding site(s), we mutated different portions of –1,024 TRE and –592 Sp1 putative binding sequences (Table 3) and tested the ability of these mutants to compete for binding as an indirect measure of their affinity for TR or Sp1. –1,024 TRE M1/M2/M3/M4 mutants did not compete for binding with labeled wild-type probe, whereas –1,024 TRE M5 mutant effectively competed for binding, suggesting that all three proposed TR elements (–1,020/–1,015, –1,014/–1,009, and –1,007/–1,002 nt) are involved in TR/DNA binding. Of the Sp1 consensus element

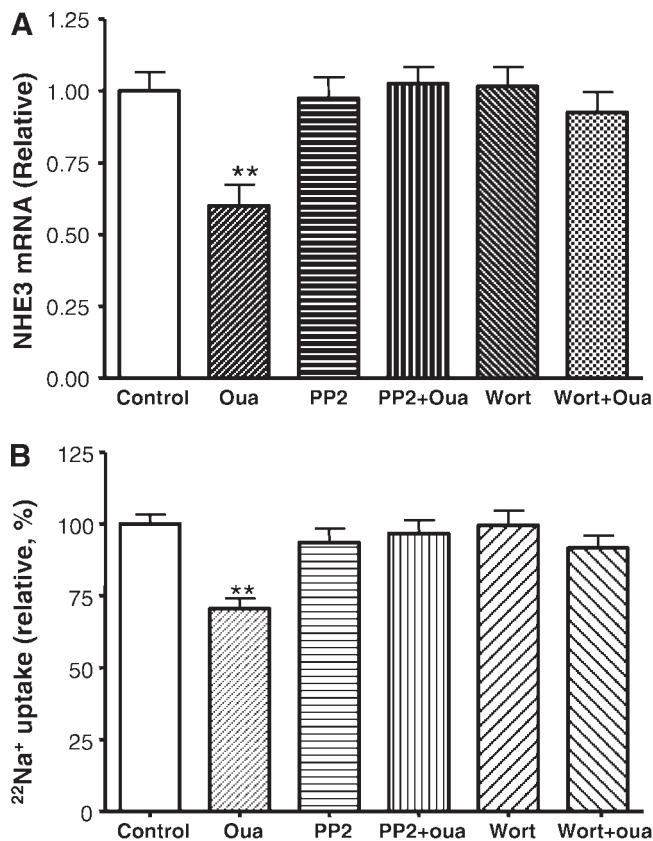


Fig. 6. c-Src and PI3K are involved in ouabain-induced downregulation of NHE3 mRNA and activity. **A:** LLC-PK₁ cells were pretreated with c-Src-specific inhibitor, PP2 (1 μ M, 30 min), or PI3K-specific inhibitor, wortmannin (Wort; 100 nM, 30 min), followed by ouabain (Oua) treatment (100 nM, 24 h) in the continued presence of a respective inhibitor. Medium with wortmannin was replaced every 4 h. NHE3 and GAPDH mRNA expression was quantified by StaRT-PCR. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 6$. **B:** H⁺-driven ²²Na⁺ uptake in LLC-PK₁ cells treated as described for A. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 3$.

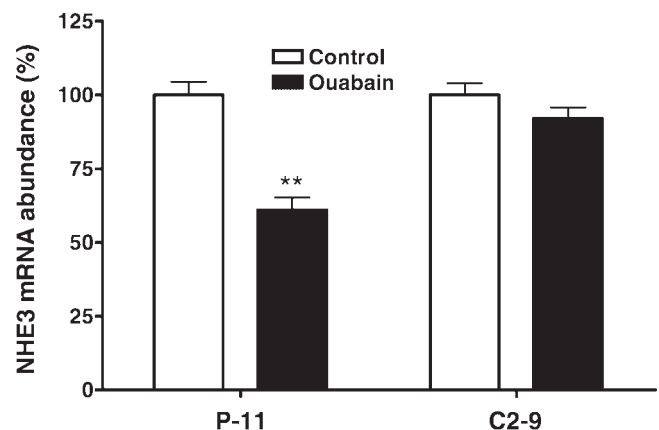


Fig. 7. Caveolin-1 is involved in ouabain-induced downregulation of NHE3 mRNA. P-11 cells (control cells transfected with an empty vector) or C2-9 cells (siRNA-mediated caveolin-1 knockdown cells) were treated with control or with 100 nM ouabain-supplemented medium for 24 h. NHE3 and GAPDH mRNA expression was quantified by StaRT-PCR. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 4$.

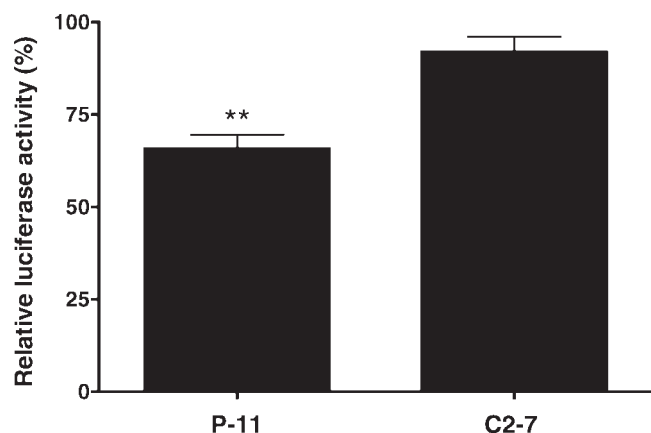


Fig. 8. Effect of ouabain treatment (100 nM for 24 h) on NHE3 promoter activity in transiently transfected control (P-11) and caveolin-deficient (C2-9) cells. Luciferase activity was normalized to protein concentration. Values are means \pm SE and are expressed relative to the control treatment. ** $P < 0.01$ ouabain vs. control, $n = 3$.

mutants, -592 Sp1 M1 mutant did not compete for binding with labeled wild-type probe, M2 competed only partially, whereas M3 mutant effectively competed for binding, suggesting that the core Sp1 binding element is located at -588/-584 nt. The identities of the transcription factors interacting with the putative ouabain-response elements (TRE and Sp) in rat cardiac fibroblasts were also confirmed by supershift assays using specific anti-TR and anti-Sp1 antibodies (data not shown).

To test whether the ouabain-induced alterations in Sp1 DNA binding postulated by the EMSA assay occur in vivo, ChIP assay was employed with control and ouabain-treated rat cardiac fibroblasts as a model. Primer sequences for PCR amplification of rat NHE3 promoter region in ChIP assay are shown in Table 2. These PCR primers were designed to amplify a sequence encompassing the identified Sp1 element and a functionally irrelevant region in the NHE3 gene promoter as a negative control (-1,365/-1,155 nt). Antibody specific for rat Sp1 was used to detect the association of this transcription factor with NHE3 gene promoter. As shown in Fig. 10, in adult rat cardiac fibroblasts, ouabain treatment (25 μ M for 24 h) significantly decreased Sp1 binding to its *cis*-element located at -588/-581 nt within the promoter region of the endogenous NHE3 gene. To test whether the effects of ouabain on Sp1 binding represent a general mechanism affecting Sp1 function, we transfected LLC-PK₁ cells with Sp1-driven pSp1-Luc reporter vector. With this approach, we failed to demonstrate a

ouabain-induced decrease in promoter activity (data not shown).

DISCUSSION

In renal epithelial cells, low concentrations of ouabain reduce transcellular Na⁺ transport without a concomitant rise in cytosolic sodium concentration (34, 36). This phenomenon has been largely attributed to interaction of ouabain with the α -subunit of Na⁺-K⁺-ATPase at the basolateral membrane, while the effects of this cardiac steroid on apical Na⁺ transport processes, although physiologically plausible, have not been explored. The present study was designed to test the hypothesis that regulation of apically expressed NHE3, a carrier with a fundamental role in Na⁺ and fluid reabsorption in the kidney, participates in the mechanism of chronic effects of low-concentration ouabain on renal sodium handling. Pig LLC-PK₁ cells, selected as a model for our studies, possess multiple characteristics of the differentiated proximal tubule epithelial cells. We previously reported that in LLC-PK₁ cells, low concentration of ouabain induces significant decrease in transcellular Na⁺ transport by a mechanism other than inhibition of the enzymatic Na⁺-K⁺-ATPase activity (36). Also, as in renal proximal tubule, dissipation of the Na⁺ gradient by ouabain inhibits H⁺ release in LLC-PK₁ cells (8).

Our results suggest that the NHE3 activity on the apical membrane of the proximal tubule epithelial cells is significantly downregulated by chronic exposure to low-concentration ouabain. This change was paralleled by a decrease in plasmalemmal NHE3 protein and mRNA abundance, a phenomenon most likely related to altered NHE3 gene transcription based on the reporter gene studies. The mechanism of ouabain action of NHE3 gene expression appears to be independent of changes in intracellular sodium concentration and is likely mediated by signaling events triggered by ouabain/Na⁺-K⁺-ATPase interaction at the basolateral membrane of LLC-PK₁ cells. This conclusion is based on the following observations: first, chronic low-concentration ouabain treatment did not alter [Na⁺]_i but reduced transcellular Na⁺ transport significantly when LLC-PK₁ monolayers were treated with ouabain in the basolateral aspect only, suggesting that the reduced apical Na⁺ entry is triggered at the basolateral membrane. Second, the observed effects of ouabain on NHE3 activity and mRNA abundance were blocked by inhibitors of c-Src (PP2) and PI3K (wortmannin), kinases previously demonstrated to play critical roles in Na⁺-K⁺-ATPase-mediated signaling. Third, the effects of ouabain on NHE3 gene promoter activity and expression of the endogenous gene were not observed in

Table 3. -1,024 TRE and Sp1 probes and mutants

TRE	
-1024 TRE probe sequence	-1024TTC CTG AAT G <u>AG GTC A</u> TA GGT GGG TTC
-1024 TRE M1	CGA TTG AAT GAG GTC ATA GGT GGG TTC
-1024 TRE M2	TTC CTA <u>GCG</u> GAG GTC ATA GGT GGG TTC
-1024 TRE M3	TTC CTG AAT <u>GAT AGT</u> ATA GGT GGG TTC
-1024 TRE M4	TTC CTG AAT GAG GTC ATA <u>TAC TGG</u> TTC
-1024 TRE M5	TTC CTG AAT GAG GTC ATA GGT <u>GGA CGT</u>
Sp1	
-592 SP1 probe sequence	-592CAC TTG <u>GCG</u> GGA GGT TTG AT
-592 SP1 M1	CGA <u>CGT</u> GCG GGA GGT TTG AT
-592 SP1 M2	CAC TTG <u>ATA TGA</u> GGT TTG AT
-592 SP1 M3	CAC TTG GCG <u>GTA TAC</u> TTG AT

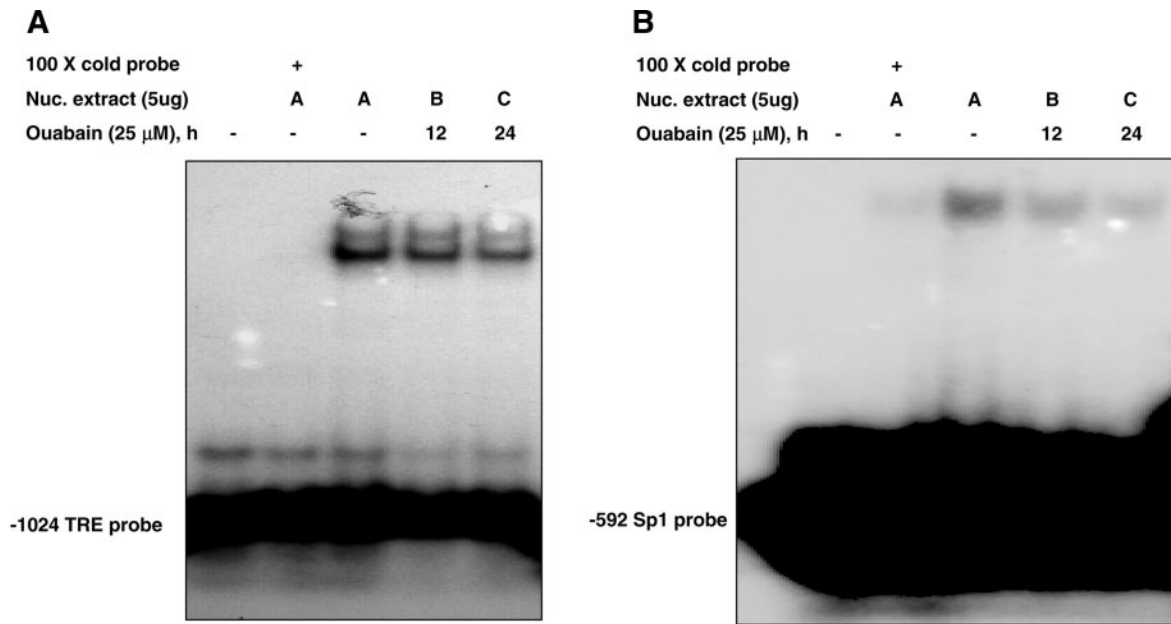


Fig. 9. Ouabain reduces TR and Sp1 binding to their respective *cis*-elements in the rat NHE3 gene promoter. Nuclear extracts from adult cardiac fibroblasts and ³²P-labeled probes were used in the EMSA assays as described in MATERIALS AND METHODS. *A*: formation of a specific complex of adult cardiac fibroblasts nuclear protein with NHE3 gene -1,024 TRE probe was decreased by ouabain treatment (25 μ M) in a time-dependent manner. A-, nuclear extract from control cells; B and C-, nuclear extracts from cells treated with ouabain for 12 or 24 h, respectively. *B*: analogous experiment with -592 Sp1 probe demonstrating time-dependent decrease in binding of Sp1 to its *cis*-element.

caveolin-1 knockdown LLC-PK₁ cells. Several previous reports support our conclusions. It has been demonstrated that ouabain-induced signal transduction involves assembly of a caveolar signaling complex (Na⁺-K⁺-ATPase signalosome) and, through a direct interaction, activation of c-Src kinase (22, 52). This mechanism is independent of changes in the intracellular ion concentration (37). It has been suggested that ouabain may induce the formation of a multiprotein complex including Na-K-ATPase, Src, EGFR, and PI3K, which then recruits AP-2 and clathrin to form clathrin-coated pits and results in the endocytosis of the enzyme into clathrin-coated vesicles, as well as early and late endosomes (34). This redistribution in response to ouabain affects both α - and β -subunit of Na⁺-K⁺-ATPase (35), whereas the involvement of the γ -modulator (FXD2) in the ATPase-mediated signaling has not been studied. The endocytosis of Na⁺-K⁺-ATPase in response to ouabain was effectively reduced by inhibitors of c-Src or PI3 kinases (35).

Interestingly, both Src and PI3 kinase activities have been implicated in regulation of NHE3-mediated H⁺ extrusion across the plasma membrane. NHE3 undergoes constitutive

endocytosis through a clathrin-coated pit pathway (10) and recycles back to the plasma membrane in a PI3K-dependent manner (31). PI3K inhibition with wortmannin or LY-294002 markedly reduced NHE3 activity and induced a pronounced loss of NHE3 from the cell surface and its accumulation in an intracellular compartment (31). The involvement of c-Src in acute regulation of NHE3 activity appears to be more complex. On one hand, c-Src kinase has been shown to be a key signal transducer in the activation of NHE3 by ANG II and by acidosis in renal epithelial cells (49, 50). In intestinal epithelial cells, however, c-Src mediates negative effects of carbachol and serotonin (18, 33). These findings suggest that ouabain may exert an acute effect on NHE3 activity via endosomal recycling, a phenomenon which could not be tested in our experimental design of long-term ouabain exposure. In these experimental settings, the primary effects of this cardiac steroid appear to be transcriptional due to highly correlated changes in NHE3 function, protein, and mRNA expression as well as NHE3 gene promoter activity. Because the kinase inhibitors applied alone did not influence NHE3 mRNA expression, yet potentially reversed the negative effects of ouabain, it is likely,

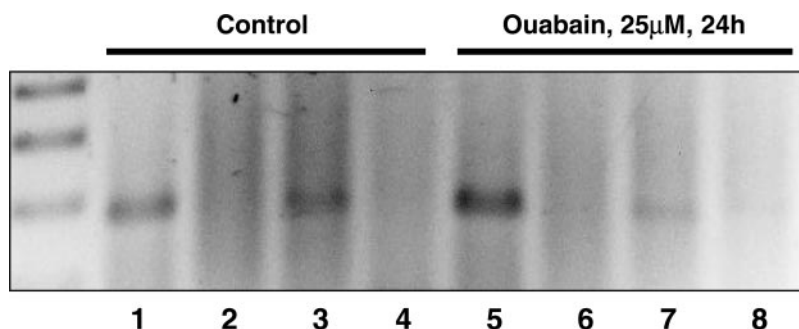


Fig. 10. Effect of ouabain on in vivo association of Sp1 transcription factor with the identified *cis*-element in the NHE3 gene promoter (-284/-81 nt region) as determined by ChIP assay. Lanes 1 and 5: input DNA; lanes 2 and 6: normal rabbit IgG; lanes 3 and 7: anti-Sp1 antibody; lanes 4 and 8: primers amplifying a functionally irrelevant region within the NHE3 gene promoter. Left: DNA ladder.

that signaling pathway(s) downstream of PI3K, c-Src and Na⁺-K⁺-ATPase endocytosis are ultimately responsible for the observed changes in NHE3 gene transcription.

The obtained data suggest an involvement of Sp1 and thyroid hormone receptor in the mechanism of ouabain-mediated decrease in NHE3 gene transcription. The TranSignal Array analyses of 54 selected transcription factors suggested that ouabain reduced binding of these two transcription factors to their respective consensus elements. Gel mobility shift assays (including supershift assay) further demonstrated that ouabain significantly decreased binding of TR and Sp1 to their cognate elements in the NHE3 promoter. These observations were verified both in renal LLC-PK₁ cells, and primary adult cardiac fibroblasts, which as described in RESULTS, expressed NHE3 and were similarly affected by ouabain treatment. Moreover, ChIP assays performed with adult rat cardiac fibroblasts demonstrated that ouabain also significantly decreases the Sp1 protein/DNA binding at the identified putative Sp1 binding motif (−588 to −581 nt) within the endogenous NHE3 gene promoter in vivo. These results strongly support the role of Sp1 and TR in ouabain-induced downregulation of NHE3 gene expression. Although recent data demonstrate an important role for the phosphorylation state of Sp1 in the regulation of multiple genes (11), it seems unlikely that c-Src or PI3K participate in the ouabain-mediated downregulation of NHE3 promoter activity by directly phosphorylating Sp1. The first of the two kinases has not been described to phosphorylate Sp1, while the only described effect of PI3K on Sp1 activity has been to increase VEGF gene transcription in response to hepatocyte growth factor (43). Additionally, the activity of the reporter gene under control of a Sp1-driven promoter (pSp1-Luc) in transiently transfected LLC-PK₁ cells was not downregulated by ouabain treatment (data not shown). This implies that the involvement of Sp1 in the response to ouabain may not represent a general mechanism. The described effects may depend on the promoter architecture and the underlying mechanism likely involves more complex protein-protein and protein-DNA interactions within the context of NHE3 5′-flanking region. A synergistic or additive action of Sp1 and thyroid hormone receptor cannot be excluded, as thyroid hormone receptor has been demonstrated to form ternary complexes including Sp1 to limit or enhance the transcriptional response (26). Because in addition to the identified Sp site, three TRE elements appear to participate in modulation of the transcriptional response of NHE3 gene to ouabain, detailed mutational studies will be necessary to define the roles for both transcription factors and their cognate *cis*-elements in this phenomenon.

To further complicate the signaling mechanism ultimately resulting in repression of NHE3 gene transcription by ouabain, one cannot exclude a transcriptional interplay with the endocytosed Na⁺-K⁺-ATPase. Our earlier observations indicate that ouabain treatment results in accumulation of the Na⁺-K⁺-ATPase α₁-subunit in the nucleus of LLC-PK₁ cells (36), where it may act as a transcriptional regulator (58). Supporting of this hypothesis is finding that the intramolecular domain (Ser692-Ser740) of the chicken α₁-subunit could function as a transcriptional regulator to regulate reporter gene expression in yeast and mammalian cells (58). This is reminiscent of a member of the EGFR family (p185neu) that could function as a transcriptional activator in both yeast and mammalian cells and could be detected in the nucleus on ligand stimulation (55).

Our results provide unequivocal evidence of ouabain-mediated changes in NHE3 expression in the renal proximal tubule and offer the first glance into the molecular mechanism of this phenomenon. Nevertheless, the precise signaling by which ouabain, a specific inhibitor and ligand of basolaterally resided Na⁺-K⁺-ATPase, triggers a decrease in apically expressed NHE3 remains largely unresolved. Based on our observations, it is conceivable that ouabain downregulates NHE3 activity and expression through its signaling function, probably relaying its signals to a different “endocytic platform” by compartmentalizing signaling molecules in the endocytic pathway. Several findings strengthen the postulated effects of cardiac steroids on renal NHE3 activity. In addition to the observations that increase of endogenous ouabain and MBG is associated with hypertension and volume expansion (17, 24), it has also been demonstrated that induction of acute hypertension in otherwise normotensive Sprague-Dawley rats alters subcellular distribution of NHE3 (retrieval of NHE3 from the apical membrane) and the Na⁺-K⁺-ATPase (retrieval of Na⁺-K⁺-ATPase from the basolateral membrane) (59, 60). While coordinated surface redistribution of NHE3 and Na⁺-K⁺-ATPase may represent the response to acute hypertension, our results add a new dimension to this regulation, whereby hypertension-related increase in circulating ouabain downregulates NHE3 gene expression, a phenomenon likely contributing to a renal compensatory response in a state of volume expansion and/or hypertension.

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