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Ouabain-stimulated trafficking regulation of the Na/K-ATPase and NHE3 in renal proximal tubule cells

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Abstract We have demonstrated that ouabain regulates protein trafficking of the Na/K-ATPase α1 subunit and NHE3 (Na/H exchanger, isoform 3) via ouabain-activated Na/K-ATPase signaling in porcine LLC-PK1 cells. To investigate whether this mechanism is species-specific, ouabain-induced regulation of the α1 subunit and NHE3 as well as transcellular $^{22}Na^{+}$ transport were compared in three renal proximal tubular cell lines (human HK-2, porcine LLC-PK1, and AAC-19 originated from LLC-PK1 in which the pig α1 was replaced by ouabain-resistant rat α1). Ouabain-induced inhibition of transcellular $^{22}Na^{+}$ transport is due to an ouabain-induced redistribution of the α1 subunit and NHE3. In LLC-PK1 cells, ouabain also inhibited the endocytic recycling of internalized NHE3, but has no significant effect on recycling of endocytosed α1 subunit. These data indicated that the ouabain-induced redistribution of the α1 subunit and NHE3 is not a species-specific phenomenon, and ouabain-activated Na/K-ATPase signaling influences NHE3 regulation.

Keywords Ouabain · Na/K-ATPase signaling · Na/K-ATPase · NHE3 · Redistribution

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Introduction

Renal sodium handling is a key determinant of long-term regulation of blood pressure [1–4]. In the kidney, the renal proximal tubules (RPTs) are responsible for more than 60 % of the net tubular Na^{+} reabsorption, mainly through basolateral Na/K-ATPase and apical NHE3. Endogenous cardiotonic steroids (CTS, also known as digitalis-like substances), which were initially classified as specific inhibitors of the Na/K-ATPase and now classified as a new family of steroid hormones, are involved in regulation of blood pressure and renal sodium handling [5–7]. Circulating CTS are markedly increased under certain conditions, such as salt loading, volume expansion, renal insufficiency, and congestive heart failure [8–10].

The pathophysiological significance of endogenous CTS has been a subject of debate since it was first proposed [11–13]. In essence, the Na/K-ATPase inhibitor (endogenous CTS) will rise in response to either a defect in renal Na^{+} excretion or high salt intake. This increase, while returning Na^{+} balance toward normal by increasing renal Na^{+} excretion, also causes increases in blood pressure by acting on the vascular Na/K-ATPase [14]. Increases in endogenous CTS regulate both renal Na^{+} excretion and blood pressure through the Na/K-ATPase [7, 14–17].

In porcine RPT LLC-PK1 cells, we have shown that ouabain inhibits active transepithelial $^{22}Na^{+}$ transport (from apical to basolateral aspect) via protein trafficking regulation of the Na/K-ATPase and NHE3 [18–20], a process requiring ouabain-activated Na/K-ATPase signaling. This novel regulatory mechanism may contribute to CTS-induced natriuresis, especially in rodents expressing the ouabain-resistant Na/K-ATPase α1 subunit [17]. In this study, we investigated if this regulatory mechanism is species-specific by characterizing the effect of ouabain on

transcellular $^{22}Na^+$ flux and redistribution of the Na/K-ATPase and NHE3. Furthermore, we also investigate the endocytic recycling (reinsertion of endocytosed protein back to plasma membrane) of internalized Na/K-ATPase and NHE3 in LLC-PK1 cells.

Experimental methods

Chemicals and antibodies

All reagents, unless otherwise mentioned, were obtained from Sigma (St. Louis, MO). Src kinase inhibitor PP2 was from CalBiochem (San Diego, CA). EZ-Kink sulfo-NHS-ss-Biotin and Immunopure immobilized streptavidin–agarose beads were obtained from Pierce Biotechnology (Rockford, IL). A rabbit polyclonal antibody against a mixture of peptides from porcine NHE3 was prepared and affinity purified [21]. Antibodies against Rab7, integrin-β1 and human NHE3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against the Na/K-ATPase α1 subunit (clone α6F) was from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Monoclonal antibodies against NHE3 (clone 4F5) and early endosome antigen-1 (EEA-1) were from Millipore Chemicon (Temecula, CA). Radioactive rubidium ($^{86}Rb^+$) and sodium ($^{22}Na^+$) were from DuPont NEN Life Science (Boston, MA).

Cell cultures

Human HK-2 cells and pig LLC-PK1 cells were obtained from the American Type Culture Collection (Manassas, VA). AAC-19 cells were generated from LLC-PK1 cells as we described previously [22]. Briefly, the ouabain-sensitive pig α1 in LLC-PK1 cells was knock-down by siRNA method. To rescue the α1 knock-down cell with ouabain-resistant rat α1 (AAC-19 cells), the α1 siRNA-targeted sequence was silently mutated to introduced rat α1 with rat α1 pRc/CMV-α1AAC expression vector. The expression of ouabain-insensitive rat α1 was selected with 3 μM ouabain in culture medium since untransfected LLC-PK1 cells are very sensitive to ouabain. Cells were cultured in Dulbecco's-modified Eagle's medium (DMEM)/F-12 mixed medium (1:1, v/v) for HK-2 or DMEM for LLC-PK1 and AAC-19, with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin) in a 5 % CO_2-humidified incubator. Culture medium was changed daily until confluency. LLC-PK1 cells and AAC-19 cells were serum-starved (in serum-free DMEM medium) for 16–18 h before treatment, and HK-2 cells were changed to medium containing 1 % FBS for 16–18 h before treatment. In assays for active transcellular $^{22}Na^+$ flux, cells were grown on transwell membrane support to form monolayer, and then treated with ouabain either in the basolateral or apical compartment. Both LLC-PK1 and AAC-19 cells can be easily grown to monolayers in DMEM medium with 10 % FBS. While HK-2 cells are hard to form monolayer with ATCC-recommended keratinocyte serum-free medium, we found that HK-2 cells could be easily grown to monolayer in DMEM/F-12 medium (with 10 % FBS) without losing its ouabain sensitivity.

Isolation of early endosome (EE) and late endosome (LE) fractions

EE and LE fractions were fractionated by a sucrose flotation gradient technique as we previously described [18, 19]. EE and LE fractions were identified with antibodies against EE marker protein EEA-1 and LE marker protein Rab7, respectively [18, 19]. In comparison with whole cell lysates, more than a tenfold enrichment of these marker proteins was observed in representative endosome fractions as we have previously shown [18].

Cell surface biotinylation

Cell surface biotinylation was conducted as we described before [18, 19]. Biotinylated proteins were pulled down with streptavidin–agarose beads, eluted with 2× Laemmli buffer (125 mM Tris–HCl, 20 % glycerol, 4 % SDS, 0.025 % bromophenol blue, 10 % 2-mercaptoethanol, pH 6.8) at 55 °C water bath for 30 min, resolved by 10 % SDS-PAGE, and then immunoblotted for the Na/K-ATPase α1 and NHE3. The same membrane was also immunoblotted with antibody against integrin-β1 to serve as loading control as described previously [17].

Ouabain-sensitive Na/K-ATPase activity assay ($^{86}Rb^+$ uptake)

For $^{86}Rb^+$ uptake assay, cells were cultured in 12-well plates and treated with or without different concentrations of ouabain for 15 min. Monensin (20 μM), a Na^+-clamping agent, was added to the medium prior to the assay to assure that the maximal capacity of active uptake was measured [23]. $^{86}Rb^+$ uptake was initiated by the addition of 1 μCi of $^{86}Rb^+$ as tracer of K^+ to each well, and the reaction was stopped after 15 min by washing four times with ice-cold 0.1 M $MgCl_2$. Trichloroacetic acid (TCA)-soluble $^{86}Rb^+$ was extracted with 10 % TCA and counted. TCA-precipitated cellular protein content was determined and used to calibrate the $^{86}Rb^+$ uptake. Data were expressed as the percentage of control $^{86}Rb^+$ uptake.

NHE3-mediated active transepithelial $^{22}Na^+$ flux and $^{22}Na^+$ uptake

Active transepithelial $^{22}Na^+$ flux (from apical to basolateral aspect of the transwell membrane supports) was performed on monolayers (grown on Costar Transwell culture filter inserts, filter pore size: 0.4 μm, Costar, Cambridge, MA) as described by Haggerty et al. [24]. Briefly, after ouabain treatment for 1 h at the concentration indicated, both apical (upper) and basolateral (lower) compartments were rinsed with ouabain-free DMEM (0 % FBS for LLC-PK1 and AAC-19 cells, and 1 % FBS for HK-2 cells). 1 ml DMEM containing $^{22}Na^+$ (1 μCi/ml) was added to the apical compartment of a filter insert, and the basolateral compartment was filled with 1 ml of DMEM. After 1 h, aliquots were removed from the basolateral compartments for scintillation counting. H^+-driven $^{22}Na^+$ uptake were determined as described by Soleimani et al. [25]. Briefly, the cells grown on 12-well plate were treated with ouabain at the concentration indicated and then washed three times with the Na^+-free buffer [in mM, 140 *N*-methyl-D-glucammonium ($NMDG^+$) Cl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.4]. The cells were then incubated for 10 min in the same Na^+-free buffer in which 20 mM $NMDG^+$ was replaced with 20 mM NH_4^+. The uptake was initiated by replacing the NH_4^+-containing buffer with Na^+-free buffer containing 1 μCi/ml $^{22}NaCl^+$. $^{22}Na^+$ uptake was stopped after 30 min by washing four times with ice-cold saline. Cell-associated radioactivity was extracted with 1 ml of 1 N sodium hydroxide, quantified by scintillation counting, and calibrated with protein content. In both experimental settings, cells were pretreated with 50 μM amiloride to inhibit amiloride-sensitive NHE1 activity.

Assessment of endocytic recycling of NHE3 and Na/K-ATPase α1 subunit in LLC-PK1 cells

Endocytic recycling of α1 and NHE3 were assessed by the method described by the Moe Laboratory [26]. Briefly, two sets of LLC-PK1 cells were biotinylated and quenched at 4 °C, and then treated with ouabain (100 nM) or vehicle (as control) for 1 h at 37 °C to induce redistribution of the Na/K-ATPase α1 and NHE3. After rinsing with ice-cold PBS-Ca–Mg (1× PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$), un-internalized surface biotinylated proteins were cleaved with 50 mM glutathione-SH (GSH-SH, a reducing agent) at 4 °C, and un-reacted free GSH-SH was oxidized by incubation with 30 mM iodoacetamide for 10 min. At this point, one set of cells was lysed with RIPA buffer to retrieve total internalized intracellular biotinylated α1 and NHE3 with streptavidin–agarose beads. Another set of cells was changed to serum-free DMEM medium and cultured at 37 °C to permit further trafficking for 2 h with or without 100 nM ouabain. Biotinylated proteins that recycled back to cell surface (reinsertion) were cleaved by GSH-SH method again. The remaining intracellular biotinylated proteins after reinsertion were retrieved with streptavidin–agarose beads, which represent the internalized biotinylated α1 and NHE3 that were not reinserted. The difference of the total intracellular α1 and NHE3, before and after reinsertion, represents the α1 and NHE3 that was internalized and then reinserted (endocytic recycling).

Western blot

Equal amounts of total protein were resolved by 10 % SDS-PAGE and immunoblotted with indicated antibodies (with dilution of 1:2,000 for the Na/K-ATPase α1 subunit and 1:1,000 dilution for NHE3, in 4 % non-fat dry milk in 1× Tris-buffered saline with 0.1 % Tween-20). The same membrane was also immunoblotted with antibodies against integrin-β1 (for surface biotinylation), EEA-1 (for EE fraction) and Rab7 (for LE fraction) to serve as loading controls (data not shown), respectively, as we previously described [17–19]. Signal detection was performed with an enhanced chemiluminescence super signal kit (Pierce, Rockford, IL). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. The signal density was determined using molecular analyst software (Bio-Rad, Hercules, CA).

Statistical analysis

Data were tested for normality (all data passed) and then subjected to parametric analysis. When more than two groups were compared, one-way ANOVA was performed prior to the comparison of individual groups with an unpaired *t* test. Statistical significance was reported at the $p < 0.05$ and $p < 0.01$ levels. SPSS software was used for all analysis (SPSS, Chicago, IL). Values are given as mean ± SE.

Results

Ouabain-mediated inhibition of the Na/K-ATPase

Ouabain-induced inhibition of the Na/K-ATPase "ion-pumping" activity (ouabain-sensitive $^{86}Rb^+$ uptake) in these RPT cell lines is summarized in Fig. 1. The IC_{50} values are consistent with the established differences of α1 ouabain sensitivity amongst these species (see "Discussion"). In LLC-PK1 cells with IC_{50} at 1 μM, 100 nM ouabain is sufficient to activate the Na/K-ATPase signaling and consequent regulation of the Na/K-ATPase and NHE3 [20]. According to the Na/K-ATPase α1 sensitivity to

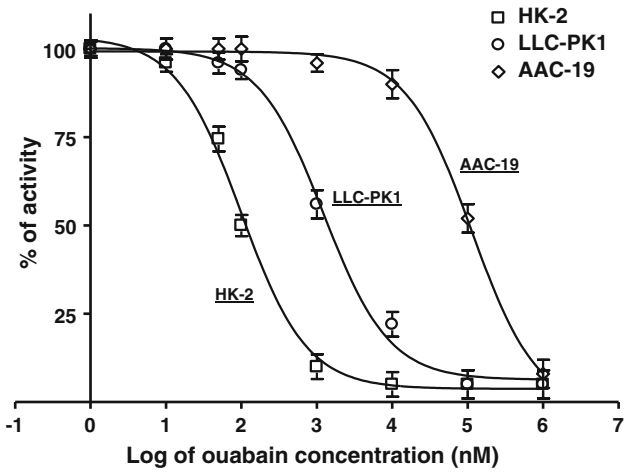

Fig. 1 Dose-dependent effects of ouabain (Oua) on Na/K-ATPase activity. The HK-2, LLC-PK1, and AAC-19 cells were grown in 12-well plates to form monolayer. The Na/K-ATPase activity (ouabain-sensitive $^{86}Rb^{+}$ uptake) was assayed as described in "Experimental methods". Data were shown as percentage of control, and each point is presented as mean ± SE of four sets of independent experiments. Curve fit analysis was performed by GraphPad software

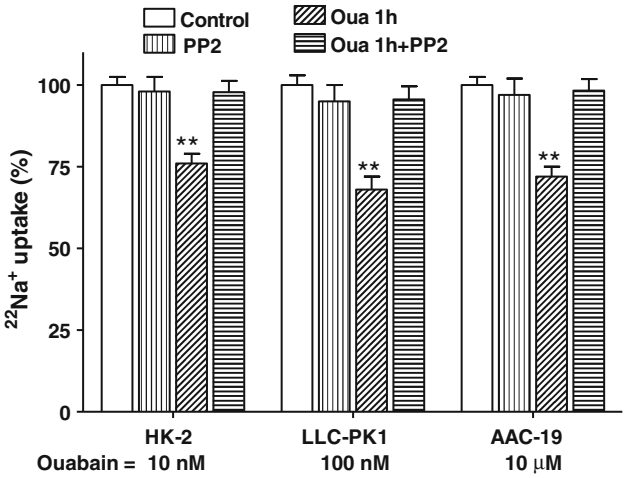

Fig. 2 Ouabain (Oua) inhibits H^{+}-driven $^{22}Na^{+}$ uptakes. The HK-2, LLC-PK1, and AAC-19 cells were grown in 12-well plates to form monolayer. After treatment with ouabain (1 h) and/or PP2 (1 μM for 30 min), $^{22}Na^{+}$ was added and assayed for H^{+}-driven $^{22}Na^{+}$ uptake. To determine H^{+}-driven Na^{+} uptake, cells were first acid loaded in Na^{+}-free buffer with 20 mM NH_4Cl and then assayed for $^{22}Na^{+}$ uptake. 50 μM amiloride was used to inhibit amiloride-sensitive NHE1 activity. Data are shown as mean ± SE, percentage of control. $n = 4$. $^{**}p < 0.01$ compared to control

ouabain, we chose ouabain concentrations that are able to activate the Na/K-ATPase signaling for these three cell lines (10 nM for HK-2, 100 nM for LLC-PK1, and 10 μM for AAC-19 cells) without significant inhibition of Na/K-ATPase activity. No significant effect on cell viability was observed when these cells were treated for 1 h with ouabain concentrations used that was evaluated by trypan blue exclusion.

Ouabain-mediated inhibition of transepithelial $^{22}Na^{+}$ flux and $^{22}Na^{+}$ uptake

We have shown that ouabain inhibits transepithelial $^{22}Na^{+}$ flux by activating Na/K-ATPase signaling in LLC-PK1 cells [18, 19]. To assess if this effect is species-specific, we measured H^{+}-driven $^{22}Na^{+}$ uptake and transepithelial $^{22}Na^{+}$ flux in these three RPT cell lines. As shown in Figs. 2 and 3, when ouabain was added in the basolateral aspect, ouabain inhibited $^{22}Na^{+}$ uptake (Fig. 2) and active transepithelial $^{22}Na^{+}$ flux (Fig. 3) in both HK-2 and AAC-19 cells in the same manner as in LLC-PK1 cells. The effect of ouabain on $^{22}Na^{+}$ flux and NHE3 activity was largely blunted when these cells were pretreated with the Src kinase inhibitor PP2 (1 μM for 30 min, at 37 °C). PP2 alone did not show significant effect. No significant inhibition of NHE3 activity was observed in all three cell lines when ouabain was added in the apical aspect (data not shown), suggesting that ouabain-induced regulation of $^{22}Na^{+}$ flux and NHE3 activity requires ouabain-activated Na/K-ATPase signaling.

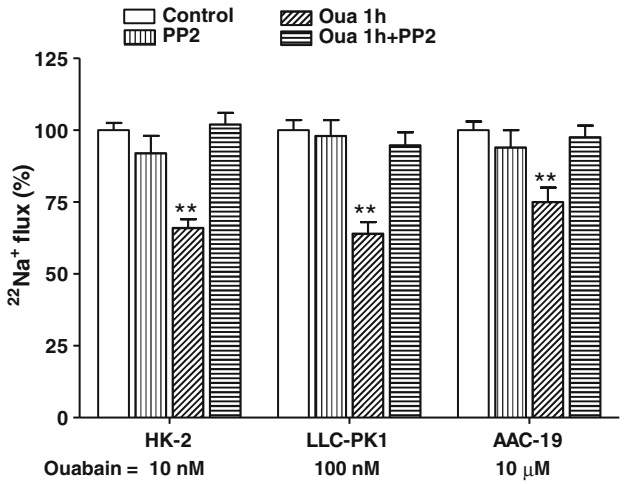

Fig. 3 Ouabain (Oua) inhibits transcellular $^{22}Na^{+}$ flux. The HK-2, LLC-PK1, and AAC-19 cells were grown in 12-well plates with transwell membrane support to form monolayer. The cells were treated with ouabain (1 h) and/or PP2 (1 μM for 30 min) in the basolateral or apical aspect. Active transepithelial $^{22}Na^{+}$ flux (apical to basolateral) was determined by counting radioactivity in the basolateral aspect at 1 h after $^{22}Na^{+}$ addition. 50 μM amiloride was added in the basolateral aspect to inhibit amiloride-sensitive NHE1 activity. Data are shown as mean ± SE, percentage of control. $n = 4$. $^{**}p < 0.01$ compared to control

Ouabain-induced protein trafficking of Na/K-ATPase and NHE3

In LLC-PK1 cells, ouabain-induced inhibition of $^{22}Na^{+}$ flux is largely due to an ouabain-mediated redistribution of

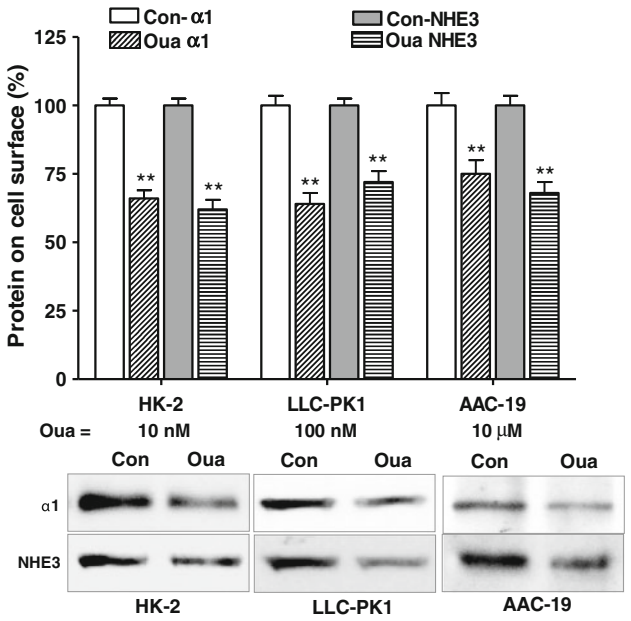

Fig. 4 Ouabain (Oua) reduces cell surface expression of the α1 and NHE3. The HK-2, LLC-PK1, and AAC-19 cells were treated with indicated concentrations of ouabain (1 h). Biotinylation of cell surface proteins was performed to assess cell surface protein contents. Data are shown as mean ± SE, percentage of control (Con). $n = 4$. $^{**}p < 0.01$ compared to control. *Inset* shows a representative western blot of four separate experiments. Immunoblotting with antibody against integrin-β1 served as loading controls (data not shown)

the Na/K-ATPase and NHE3 via Na/K-ATPase signaling. To further test this regulatory mechanism, these three cell lines were treated with or without ouabain to assess the ouabain-induced redistribution. As shown in Fig. 4, the ouabain-induced redistribution of the α1 subunit and NHE3 was caused by a decrease in cell surface α1 subunit and NHE3 in both HK-2 and AAC-19 cells (as we previously reported in LLC-PK1 cells [18, 19]). Pretreatment with PP2 abolished ouabain-induced redistribution of the α1 subunit and NHE3 (data not shown). As shown in Table 1, ouabain (1 h) caused a dose-dependent reduction of cell surface α1 subunit and NHE3. Furthermore, the ouabain-induced reduction of cell surface α1 subunit and NHE3 was closely correlated to ouabain-induced inhibition of transcellular $^{22}Na^{+}$ flux and NHE3 activity (Figs. 2, 3).

Ouabain-mediated regulation of endocytic recycling of the Na/K-ATPase α1 subunit and NHE3 in LLC-PK1 cells

Endocytic recycling is essential for maintaining the distinction between apical and basolateral membranes in polarized cells, even though the recycling pathways may be redundant [27]. It has been shown that ouabain redistributes the Na/K-ATPase into both LEs and lysosomes [28], presumably for degradation. To explore the underlying mechanism, we used LLC-PK1 cells to assess endocytic recycling of the α1 subunit and NHE3. As shown in Fig. 5a, GSH-SH (a reducing agent) was able to cleave over 90 % of protein-bound biotin. Ouabain (100 nM, 1 h) accumulated the α1 subunit (control 100 ± 9.6 % vs. ouabain 198.3 ± 18.2, $n = 3$, $p < 0.01$) and NHE3 (control 100 ± 8.9 % vs. ouabain 187.4 ± 16.5, $n = 3$, $p < 0.01$) in intracellular compartments (Fig. 5b) after cleavage of surface protein-bound biotin with GSH-SH method. After 2 h-period of recycling of internalized biotinylated proteins, recycled biotinylated proteins were cleaved again with GSH-SH. The total intracellular biotinylated α1 and NHE3 after recycling, which represent unrecycled biotinylated α1 and NHE3, are shown in Fig. 5c. The reinsertion (endocytic recycling) was presented as the difference of the total intracellular α1 and NHE3 before and after reinsertion (Fig. 5d). The present data indicate that ouabain treatment not only induced NHE3 redistribution, but it also inhibited the endocytic recycling of NHE3. Interestingly, the endocytic recycling of the α1 subunit was not significantly affected by ouabain. These observations suggested that, while most of the internalized α1 was destined for degradation, at least part of internalized NHE3 was recycled back to cell membrane surface. Despite the lack of microvilli in cultured renal proximal tubular cells [29], our observation is reminiscent of the model of NHE3 moving along the microvilli structure [30].

To further explore the destination of the α1 subunit and NHE3, we determined the protein content of these two transporters in EE and LE fractions in response to ouabain. As shown in Fig. 6, ouabain treatment (100 nM, 1 h) stimulated accumulation of both α1 and NHE3 in EE fractions. However, ouabain significantly accumulated α1, but not NHE3 in LE fractions. The NHE3 protein content in LE fractions was not significantly increased after ouabain treatment, even in the presence of the lysosomotropic weak base agent chloroquine (0.2 mM, pretreated for 2 h) which inhibits degradation by the lysosomal pathway. On the other hand, pretreatment with chloroquine caused a further accumulation of the α1 subunit in LE fractions in response to ouabain, suggesting that the endocytosed α1 subunit, but not NHE3, is largely degraded through the LE/lysosome pathway.

Discussion

The renal tubular Na/K-ATPase comprises a final site for the regulation of renal sodium transport by many factors [31]. Our recent work indicates that ouabain is one of these factors that acting via a coordinated regulation of the Na/K-ATPase and NHE3 through Na/K-ATPase signaling

Table 1 Ouabain causes a dose-dependent inhibition of transcellular $^{22}Na^+$ flux, $^{22}Na^+$ uptake, and surface α1 and NHE3

	HK-2 cells				
	Ouabain (nM) for 1 h				
	0	1	5	10	50
$^{22}Na^+$ flux	100 ± 4.5	102.1 ± 3.8	81.2 ± 5.2[a]	68.5 ± 4.8[b]	61.8 ± 5.9[b]
$^{22}Na^+$ uptake	100 ± 5.1	98.4 ± 4.2	83.6 ± 5.6[a]	76.6 ± 6.2[b]	67.8 ± 5.3[b]
Surface α1	100 ± 6.1	96.5 ± 6.7	84.2 ± 6.2[a]	63.5 ± 7.1[b]	59.7 ± 7.4[b]
Surface NHE3	100 ± 4.5	99.2 ± 6.1	86.2 ± 6.8[a]	67.3 ± 7.1[b]	69.1 ± 7.9[b]
	LLC-PK1 cells				
	Ouabain (nM) for 1 h				
	0	10	25	100	1,000
$^{22}Na^+$ flux	100 ± 5.2	99.1 ± 4.8	82.4 ± 7.4[a]	65.5 ± 6.8[b]	60.2 ± 8.6[b]
$^{22}Na^+$ uptake	100 ± 5.8	95.7 ± 6.7	88.3 ± 8.1[a]	64.8 ± 7.2[b]	58.5 ± 7.8[b]
Surface α1	100 ± 7.9	97.3 ± 8.1	85.6 ± 7.2[a]	65.2 ± 7.7[b]	67.6 ± 8.4[b]
Surface NHE3	100 ± 6.5	98.6 ± 8.8	83.2 ± 7.4[a]	72.7 ± 6.1[b]	69.3 ± 7.6[b]
	AAC-19 cells				
	Ouabain (nM) for 1 h				
	0	100	1,000	10,000	25,000
$^{22}Na^+$ flux	100 ± 6.2	96.3 ± 3.8	86.4 ± 7.3[a]	75.5 ± 6.9[b]	68.8 ± 7.6[b]
$^{22}Na^+$ uptake	100 ± 7.2	97.8 ± 7.1	85.1 ± 8.5[a]	71.6 ± 8.1[b]	65.2 ± 9.6[b]
Surface α1	100 ± 8.2	96.5 ± 7.6	87.4 ± 8.1[a]	75.2 ± 7.9[b]	67.4 ± 9.1[b]
Surface NHE3	100 ± 9.1	94.7 ± 8.7	84.8 ± 9.3[a]	69.5 ± 8.8[b]	61.8 ± 9.9[b]

$^{22}Na^+$ flux and $^{22}Na^+$ uptake were measured as described in "Experimental methods". Surface α1 and NHE3 were determined by cell surface biotinylation. $N = 3$ for each treatment

[a] $p < 0.05$ compared to controls

[b] $p < 0.01$ compared to controls

[17–20]. In this study, we have investigated whether this ouabain-mediated regulatory model is species-specific. Our present study indicated that ouabain-induced regulation of the Na/K-ATPase and NHE3 is not species-specific. First, ouabain was able to inhibit the active transepithelial $^{22}Na^+$ flux in these three cell lines. This effect was largely prevented by blocking c-Src activation with PP2 pretreatment. Secondly, ouabain-induced redistribution and reduction of the cell surface Na/K-ATPase and NHE3 contributed to the inhibition of active $^{22}Na^+$ flux. Third, the effect of ouabain on active $^{22}Na^+$ flux was only observed when ouabain was applied in the basolateral, but not in the apical aspect. Fourth, the ouabain-induced reduction of the cell surface α1 subunit contributed to the ouabain-induced inhibition of $^{22}Na^+$ flux and $^{22}Na^+$ uptake. Taken together, these observations suggest that the ouabain-induced regulation of the Na/K-ATPase and NHE3 is not species-specific, and that the Na/K-ATPase is the functional receptor for ouabain-induced regulation.

Activation of c-Src is critical in the ouabain-activated Na/K-ATPase signaling and redistribution of the Na/K-ATPase and NHE3 [18–21, 32]. When compared with HK-2 and LLC-PK1 cells, a higher concentration of ouabain was needed to regulate activity and redistribution of the Na/K-ATPase and NHE3 in AAC-19 cells expressing native pig NHE3 and rat α1. This is consistent with the established differences in ouabain sensitivity of the α1 subunit as well as ouabain-stimulated c-Src activation between these two cell lines [22]. It is well known that there are large differences in sensitivity of the Na/K-ATPase to ouabain based on α isoforms and species [33–35]. Specifically, the rodent α1 is far less sensitive than pig, dog, or human α1. Higher concentrations of ouabain were required to activate Na/K-ATPase signaling in rodents, compared to other species [22, 36–39]. We have also shown that a higher concentration of ouabain (10 μM) is needed to activate c-Src in isolated renal proximal tubules of Dahl salt-resistant rats [17]. Most interestingly, different natriuretic responses were observed between transgenic mice expressing ouabain-sensitive α1 and wild-type mice expressing ouabain-resistant α1 [16] in which the ouabain binding site of the α1 subunit plays a critical role [40]. As shown in Table 1, the ouabain sensitivity of the α1 subunit influenced the ouabain-induced inhibition of $^{22}Na^+$ flux as well as surface reduction of the α1 subunit and NHE3. This study further suggests that species-specific α1 sensitivity to ouabain might explain the species differences in ouabain-induced natriuresis in vivo [7, 10, 16, 41]. Considering the high ouabain sensitivity of human α1 subunit, this could explain how pathophysiological circulating CTS might affect renal sodium handling, especially in the view that endogenous ouabain is a natriuretic hormone and has a

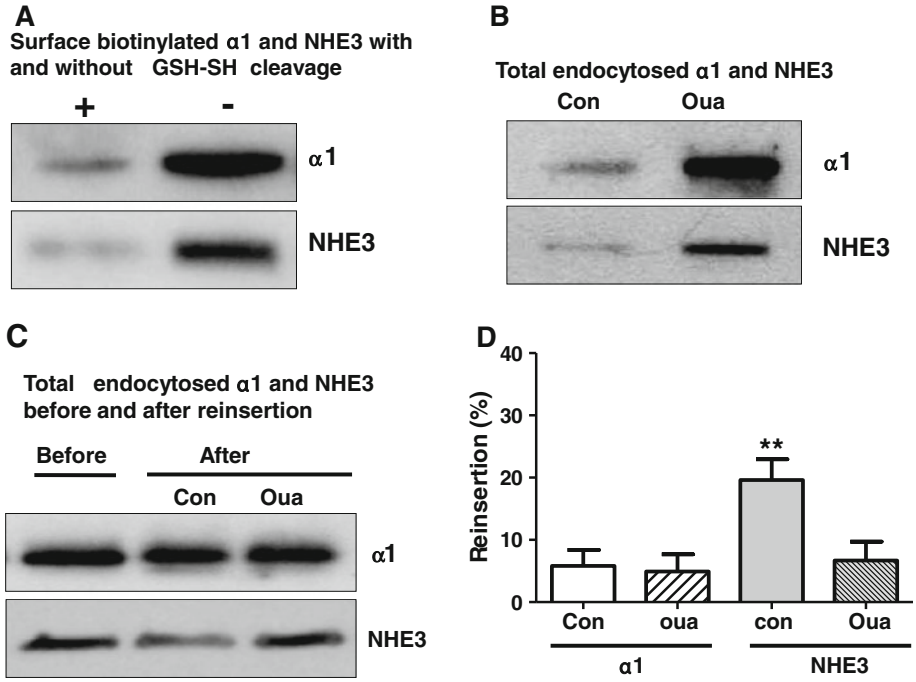

Fig. 5 Ouabain (Oua) inhibites endocytic recycling of NHE3 in LLC-PK1 cells. The experiments were performed as described in "Experimental methods". **a** Determination of GSH cleavage efficiency. Cell surface proteins were biotinylated and applied with or without GSH cleavage procedure. **b** Retrieval of total internalized biotinylated α1 and NHE3 after treatment with or without ouabain (100 nM, 1 h) and GSH cleavage. **c** Assessment of the effect of ouabain on reinsertion. **d** The graph bars represented reinsertion of endocytosed α1 and NHE3, the difference of total endocytosed α1 and NHE3 before and after resinsertion procedure. $n = 3$. $^{**}p < 0.01$

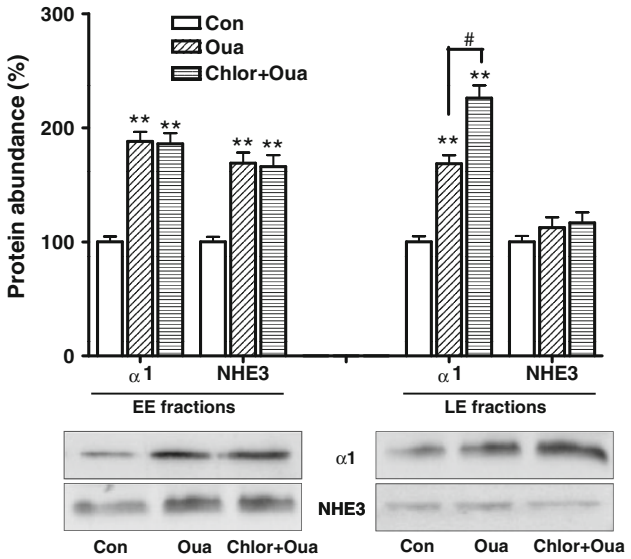

Fig. 6 Ouabain (Oua) accumulates the Na/K-ATPase α1 subunit, but not NHE3 in LE in LLC-PK1 cells. LLC-PK1 cells were treated with or without ouabain (100 nM for 1 h), with or without pretreatment of chloroquine (chlor, 0.2 mM, pretreated for 2 h). EE and LE fractions were isolated at the end of ouabain treatment. Equal amount of proteins (25 μg) was used to determine protein contents of the α1 and NHE3 by western blot analysis. $n = 4$. $^{**}p < 0.01$ compared to controls from EE and LE, respectively. $\#p < 0.01$, comparison of α1 subunit in LE fraction with or without chloroquine pretreatment. *Inset* shows a representative western blot of four separate experiments. Immunoblotting with antibodies against EEA-1 (for EE fractionation) and Rab7 (for LE fractionation) served as loading controls, respectively (data not shown)

physiological role in controlling sodium homeostasis in normal rats [7].

After the redistribution of membrane proteins, subsequent intracellular trafficking differs among different endocytosed proteins. Receptor-mediated redistribution is believed to be an effective pathway to reduce cell surface signaling receptors. In our experimental settings, ouabain caused accumulation of the α1 subunit in LE fractions, but failed to affect its endocytic recycling (Figs. 5, 6). This is a reminiscence of the early observation that ouabain caused the redistribution of the Na/K-ATPase into LEs and lysosomes [28], suggesting that the endocytosed Na/K-ATPase is most likely degraded in LE/lysosome pathway. On the other hand, the cell surface expression of NHE3 is likely reduced by inhibition of the recycling of internalized NHE3 (Figs. 5, 6). However, the mechanism is not clear. In both cases, we cannot exclude the possibility that both recycling and degradation were affected by ouabain, but the overall observed effect was a balance between these two processes.

Renal Na^+ reabsorption through NHE3 plays an important role in salt sensitivity, as well as in the development and control of sodium homeostasis and blood pressure [42–45]. Recently, we have demonstrated that impaired Na/K-ATPase-Src signaling contributes to salt sensitivity in Dahl rats [17]. This is consistent with the observation that a high salt diet stimulates redistribution of RPT Na/K-ATPase and NHE3 [46]. Although the mechanisms are still being elucidated, accumulating evidence supports the notion of

coordinated regulation of the Na/K-ATPase and NHE3 [47]. It appears that the pathways regulating the Na/K-ATPase and NHE3 are numerous and redundant, and CTS-induced coordinated regulation is one of the pathways that occur in response to conditions that cause an increase in endogenous CTS.

In summary, our data indicate that the Na/K-ATPase is the functional receptor for ouabain-induced regulation of the Na/K-ATPase and NHE3 (and thus transcellular Na^+ transport), as we previously proposed [20]. This regulation is not species-specific, but the species-specific α1 ouabain sensitivity may partially account for the species differences observed in ouabain-induced natriuresis [7, 10, 16, 41]. In ouabain-induced trafficking regulation, endocytic recycling of internalized NHE3, but not the α1 subunit, was inhibited by ouabain.

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