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# Involvement of Reactive Oxygen Species in a Feed-forward Mechanism of Na/K-ATPase-mediated Signaling Transduction*

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**Background:** Na/K-ATPase signaling regulates sodium reabsorption in renal proximal tubules.
**Results:** Carbonylation modification of the Na/K-ATPase $\alpha$1 subunit regulates Na/K-ATPase signaling and subsequent transepithelial sodium transport.
**Conclusion:** ROS is involved in the Na/K-ATPase signaling transduction in a feed-forward mechanism.
**Significance:** ROS regulates Na/K-ATPase signaling and sodium transport in LLC-PK1 cells.

**Cardiotonic steroids (such as ouabain) signaling through Na/K-ATPase regulate sodium reabsorption in the renal proximal tubule. We report here that reactive oxygen species are required to initiate ouabain-stimulated Na/K-ATPase·c-Src signaling. Pretreatment with the antioxidant *N*-acetyl-L-cysteine prevented ouabain-stimulated Na/K-ATPase·c-Src signaling, protein carbonylation, redistribution of Na/K-ATPase and sodium/proton exchanger isoform 3, and inhibition of active transepithelial $^{22}Na^+$ transport. Disruption of the Na/K-ATPase·c-Src signaling complex attenuated ouabain-stimulated protein carbonylation. Ouabain-stimulated protein carbonylation is reversed after removal of ouabain, and this reversibility is largely independent of *de novo* protein synthesis and degradation by either the lysosome or the proteasome pathways. Furthermore, ouabain stimulated direct carbonylation of two amino acid residues in the actuator domain of the Na/K-ATPase $\alpha$1 subunit. Taken together, the data indicate that carbonylation modification of the Na/K-ATPase $\alpha$1 subunit is involved in a feed-forward mechanism of regulation of ouabain-mediated renal proximal tubule Na/K-ATPase signal transduction and subsequent sodium transport.**

The Na/K-ATPase $\alpha$1 subunit directly interacts with c-Src kinase via two pairs of domain interactions to form a functional receptor complex (1, 2), *i.e.* the Na/K-ATPase·c-Src signaling complex. The Na/K-ATPase $\alpha$1 subunit provides the ligand-binding site, and the associated c-Src functions as the kinase moiety, amplifying and converting the binding signal to the stimulation of multiple protein kinase cascades, including c-Src and PI3K. In addition, ROS[2] generation is an integrated component of Na/K-ATPase signaling. Ouabain stimulates a Ras-dependent ROS generation via Na/K-ATPase signaling (3, 4), and increases in ROS induced by glucose oxidase (GO) stimulate Na/K-ATPase endocytosis (5). Increases in oxidative stress inhibit Na/K-ATPase activity and promote its susceptibility to degradation (6, 7). Furthermore, oxidative modifications, such as glutathionylation of cysteine residue(s) of the Na/K-ATPase $\beta$1 subunit (8) and $\alpha$ subunit (9), inhibit Na/K-ATPase activity, by either stabilizing the enzyme in an E2-prone conformation or by blocking the ATP-binding site.

Recently, we reported that CTS, signaling through the Na/K-ATPase, inhibits renal proximal tubule (RPT)-mediated sodium reabsorption and thus increases sodium excretion to counterbalance sodium retention and the related blood pressure increase (10–16). Impairment of the RPT Na/K-ATPase·c-Src signaling contributes to experimental Dahl salt-sensitive hypertension (16). However, there is no difference in the Na/K-ATPase $\alpha$1 subunit gene (*Atp1a1*) coding (17), ouabain sensitivity (18), and expression (16) between the Dahl salt-resistant and salt-sensitive rats (Jr strains). Moreover, acute salt loading causes higher plasma CTS levels in the salt-sensitive rat when compared with the salt-resistant rat (19). These observations indicate the presence of other regulatory factor(s) that regulate Na/K-ATPase signaling. We report here that protein carbonylation of the Na/K-ATPase $\alpha$1 subunit actuator (A) domain is involved in RPT Na/K-ATPase signal transduction in a feed-forward mechanism.

## EXPERIMENTAL PROCEDURES

*Chemicals and Antibodies*—All chemicals, except as mentioned otherwise, were obtained from Sigma. Proteasome

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[2] The abbreviations used are: ROS, reactive oxygen species; CTS, cardiotonic steroids; EE, early endosome; GO, glucose oxidase; LLC-PK1, pig-originated proximal tubule cell line; NAC, *N*-acetyl-L-cysteine; NHE3, sodium/proton exchanger isoform 3; DNPH, 2,4-dinitrophenylhydrazine; DNP, 2,4-dinitrophenyl; DHE, dihydroethidium; t-Src, total c-Src; RPT, renal proximal tubule.

inhibitor MG132 and Src kinase inhibitor PP2 were from EMD Chemicals-Calbiochem. Monoclonal antibodies against the Na/K-ATPase α1 subunit (clone α6F and clone C464.6) were from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA) and EMD Millipore Upstate (Billerica, MA), respectively. Monoclonal antibody against early endosome antigen-1 (EEA-1) was from EMD Millipore Chemicon (Temecula, CA). Polyclonal anti-Src (Tyr(P)$^{418}$) phosphospecific antibody and membrane-permeable dihydroethidium (DHE) were from Invitrogen. Monoclonal antibody against total c-Src was from Santa Cruz Biotechnology (Santa Cruz, CA). 2,4-Dinitrophenylhydrazine (DNPH) and antibody against 2,4-dinitrophenyl (DNP) hydrazone derivatives were from Sigma. Radioactive $^{22}Na^+$ was from PerkinElmer Life Sciences.

*Cell Cultures*—Porcine RPT cell line LLC-PK1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured with DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin, in a 5% $CO_2$-humidified incubator. Culture medium was changed daily until confluence. Cells were serum-starved for 16–18 h before treatment. In assays for active transcellular $^{22}Na^+$ flux, cells were grown on Transwell® membrane support (Costar Transwell® culture filter inserts, filter pore size 0.4 μm, Costar, Cambridge, MA) to form monolayers. To test the possible impact of serum starvation on the effect of ouabain and GO, some experiments were performed without serum starvation to test the effect of 10% FBS on ouabain- and GO-induced c-Src activation and protein carbonylation.

Src kinases (Src, Yes, and Fyn)-null SYF and SYF + c-Src (SYF cells overexpressing c-Src) cells were also obtained from the ATCC. SYF and SYF + c-Src cells were generated from mouse embryo fibroblasts and cultured in DMEM with 10% FBS. Medium was changed daily until the cells reached 80–90% confluence, at which time the medium was changed to DMEM with 1% FBS for 16–18 h before experiments.

*Detection of Superoxide by Fluorescence Microscopy*—DHE was used as a superoxide probe to determine DHE fluorescence, following the procedure (20) with minor modification. Briefly, after treatment with ouabain (100 nM, 1 h) and rinsing with 1× PBS, LLC-PK1 cells were incubated with DHE (5 μM, 20 min) and then washed with 1× PBS (three times for 5 min each) in a dark chamber on a rocker. For each set, DHE staining and image acquisition of control and ouabain-treated cells were performed in parallel. Images (four to six per slide) were acquired by using an Olympus FSX100 box type fluorescence imaging device (Olympus America Inc., Center Valley, PA) with fixed parameters for all samples. Background fluorescence was estimated by an image in an area free of cells. Fluorescence intensity (minus background fluorescence in the same slide) was analyzed by ImageJ software (version 1.32j, National Institutes of Health) and normalized to the number of cells showing DHE-positive staining.

*Isolation of Early Endosome (EE) Fractions*—The EE fractions (EEA-1-positive) were isolated by sucrose floatation centrifugation and identified previously as described (13, 21). The enrichment of EE fractions was assessed by the EE marker EEA-1. Equal amounts of total protein from the EE fraction of each sample was precipitated with trichloroacetic acid for Western blot.

*Active Transepithelial $^{22}Na^+$ Flux Assay*—LLC-PK1 cells were cultured on Transwell® membrane support to form monolayers and then treated with ouabain or GO for 1 h either in a basolateral or apical compartment. Active transepithelial $^{22}Na^+$ flux (from apical to basolateral compartment) was determined by counting radioactivity in the basolateral aspect 1 h after $^{22}Na^+$ addition to the apical compartment as described previously (13). Each experiment was performed in triplicate. Cells were pretreated with 50 μM amiloride for 30 min to inhibit amiloride-sensitive NHE1 activity.

*Measurement of c-Src Phosphorylation and Interaction between c-Src and Na/K-ATPase α1 Subunit*—Whole cell lysates were prepared with Nonidet P-40 buffer (containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaCl, 50 mM HEPES, 10% glycerol (pH 7.4), 1 mM sodium vanadate, 0.5 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride, and protease inhibitor mixture for general use (Sigma)). Activation of c-Src and interaction between c-Src and the α1 subunit were determined as described previously (22). After clarification by centrifuge, 300 μg of total protein was immunoprecipitated with antibody against total c-Src and protein G-agarose beads (EMD Millipore Upstate) and then eluted with 2× Laemmli buffer. After immunoblotting for phospho-c-Src and α1 subunit, the same membrane was stripped and immunoblotted for total c-Src (t-Src). Activation of c-Src was expressed as the ratio of phospho-c-Src/t-Src with both measurements normalized to 1.0 for the control samples. Interaction between the α1 subunit and c-Src was expressed as the ratio of α1/t-Src with both measurements normalized to 1.0 for the control samples.

*Assessment of Protein Carbonylation*—Whole cell lysates were prepared with Nonidet P-40 buffer as described above. Equal amounts of total protein from each sample were denatured with 6% SDS (final concentration), derivatized with 1× DNPH (freshly diluted with distilled water from 10× DNPH stock solution, 100 mM in 100% trifluoroacetic acid) to form DNP hydrazone derivatives, and then neutralized with neutralization buffer (30% of glycerol in 2 M Tris). This was followed by either Western blotting for protein carbonylation assay in whole cell lysates or immunoprecipitation-DNP studies. For immunoprecipitation-DNP, neutralized DNP derivatives were reacted with anti-DNP antibody, precipitated with protein G-agarose beads, and then eluted with 2× Laemmli buffer. Eluents were immunoblotted with antibodies against Na/K-ATPase α1 subunit, NHE3, and c-Src.

To assess whether ouabain-induced protein carbonylation is reversible, LLC-PK1 cells were treated with either ouabain or GO for 1 h to induce protein carbonylation. Control and ouabain-treated cells were collected at this point (wash = 0). In another set of ouabain-treated cells, unbound ouabain was removed (by extensive washing with culture medium) and further cultured in ouabain-free medium for 2 h (wash = 2). Protein carbonylation in whole cell lysates was determined and compared with control and without washed ouabain-treated cells.

Membrane Ponceau S staining was used for loading control of the protein carbonylation studies because we could not iden-

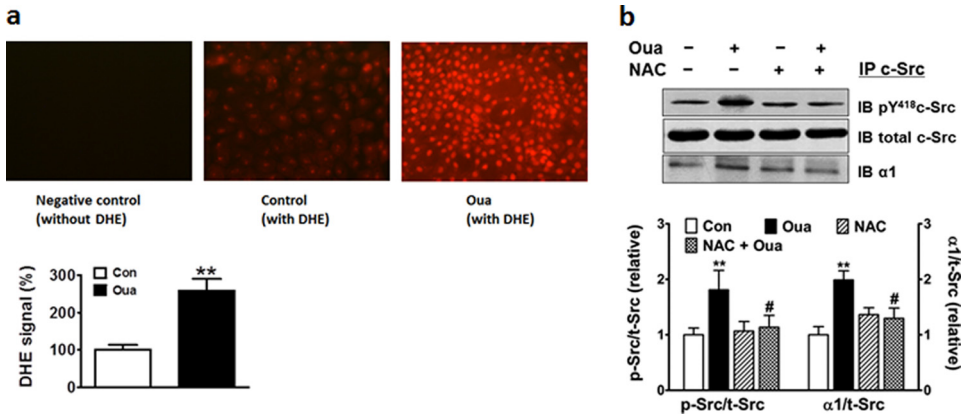



FIGURE 1. **ROS is required in ouabain-induced activation of the Na/K-ATPase signaling.** *a,* set of representative DHE staining images showing negative control (without DHE), control (with DHE), and ouabain (100 nM, 1 h) treatment (with DHE). Quantitative analysis (*bar graph*) showed that ouabain (*Oua*) significantly stimulated superoxide-related ROS generation. $n = 6$, **, $p < 0.01$ *versus* control (*Con*). *b,* LLC-PK1 cells were pretreated with NAC (10 mM, 30 min) before ouabain (100 nM, 15 min) treatment. Immunoprecipitation (*IP*) against total c-Src was performed to determine Tyr(P)$^{418}$ c-Src phosphorylation and its association with the Na/K-ATPase $\alpha$1 subunit. A representative Western blot and quantitative analysis are shown. The phosphorylation of c-Src was expressed as the ratio of phosphorylated c-Src (*p-Src*) *versus* t-Src. The association between total c-Src and the $\alpha$1 subunit was expressed as the ratio of the $\alpha$1 *versus* c-Src. $n = 4$. **, $p < 0.01$ *versus* control, and #, $p < 0.01$ *versus* ouabain treatment. *IB*, immunoblot.

tify an uncarbonylated protein under the experimental conditions. To quantify the carbonylation level, optical signal densities of the protein bands from each lane were quantified. The signal density values of control samples were normalized to 1.0 with Ponceau S staining as loading control.

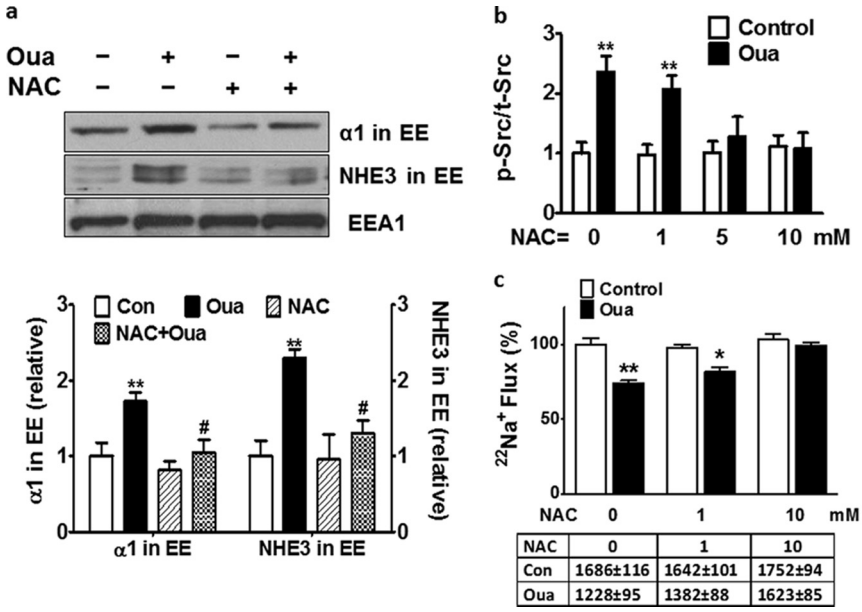
*Identification of Protein Carbonylation Site(s) by LC-MS/MS*—Protein identification was performed in the mass spectrometry-based proteomics Facility (Department of Pathology, University of Michigan), using previously described protocols (23). LLC-PK1 cells were treated with either ouabain (100 nM) or GO (3 milliunits/ml) for 1 h. The Na/K-ATPase $\alpha$1 subunit was immunoprecipitated with monoclonal anti-$\alpha$1 antibody (clone C464.6, EMD Millipore) and separated by SDS-PAGE. Coomassie Brilliant Blue staining was performed using mass spectrometry-compatible NOVEX Coomassie Blue colloidal staining (Invitrogen) as instructed by the manufacturer. The $\alpha$1 bands were excised and processed for in-gel trypsin digestion with sequencing grade modified trypsin (Promega). Resulting peptides were resolved on a nano-capillary reverse phase column (Picofrit column, New Objective) and directly introduced into a linear ion-trap mass spectrometer (LTQ Orbitrap XL, Thermo Fisher). LC-MS/MS analysis was operated in a dual play mode where it was set to collect one full scan (MS) followed by data-dependent, collision-induced dissociation spectra (MS/MS). The spectra data were searched against porcine protein database. Proteins and peptides were identified by comparing the data against database appended with decoy (reverse) sequences using the X!Tandem/Trans-Proteomic Pipeline (TPP) software suite (24–26). Precursor and fragment mass tolerance were set to 50 ppm and 0.8 Da, respectively. Oxidation of proline to glutamic semialdehyde ($\Delta m = +15.9949$ Da), threonine to 2-amino-3-ketobutyric acid ($\Delta m = -2.0156$ Da), lysine to aminoadipic semialdehyde ($\Delta m = -1.0316$ Da), and arginine to glutamic semialdehyde ($\Delta m = +43.0534$ Da), indicators of direct carbonylation, were considered as potential direct carbonylation modifications. The modified peptide was identified with high confidence (PeptideProphet probability of >0.95) with spectral counts (which counts the number of spectra identified for a given peptide in different biological samples). All proteins with a probability score of >0.95 (false discovery rate <1%) were considered positive identifications, and the collision-induced dissociation spectra of peptides with modifications were manually verified.

*Western Blotting*—For Western blot analysis, equal amounts of total protein were resolved by 10% SDS-PAGE, transferred onto the PVDF membrane (EMD Millipore), and immunoblotted with the indicated antibodies. Signal detection was performed with an enhanced chemiluminescence SuperSignal kit (Pierce). Multiple exposures were analyzed to ensure that the signals were within the linear range of the film. The signal density was determined using Molecular Analyst software (Bio-Rad).

*Statistical Analysis*—Data were tested for normality and then subjected to parametric analysis. When more than two groups were compared, one-way analysis of variance was performed prior to comparison of individual groups, and the post hoc *t* tests were adjusted for multiple comparisons using Bonferroni's correction. Statistical significance was reported at the $p < 0.05$ and $p < 0.01$ levels. SPSS software was used for all analysis. Values are given as mean ± S.E.

## RESULTS

*Role of ROS in Ouabain-induced Na/K-ATPase·c-Src Signaling, Transporter Trafficking, and Inhibition of Transepithelial $^{22}Na^+$ Flux*—To test whether ouabain induced ROS generation in LLC-PK1 cells, we used DHE as a superoxide probe. Ouabain (100 nM, 1 h) significantly increased DHE fluorescent signaling (Fig. 1*a*), suggesting that ouabain stimulated a superoxide-related ROS generation. To evaluate the role of ROS in the signaling function of the Na/K-ATPase, we used the antioxidant *N*-acetyl-L-cysteine (NAC) to eliminate ROS increases. Pretreatment with NAC (10 mM, 30 min) significantly ($p < 0.01$) attenuated ouabain (100 nM, 15 min)-stimulated c-Src activation and interaction between the $\alpha$1 subunit and c-Src (Fig. 1*b*). NAC alone had no effect on phosphorylation of c-Src as well as $\alpha$1/c-Src interaction.




| NAC | 0 | 1 | 10 |
|---|---|---|---|
| Con | 1686±116 | 1642±101 | 1752±94 |
| Oua | 1228±95 | 1382±88 | 1623±85 |

FIGURE 2. **ROS is required in ouabain-induced redistribution of the Na/K-ATPase α1 subunit and NHE3 and inhibition of transcellular $^{22}Na^+$ flux.** *a,* representative Western blot and quantitative analysis showing that pretreatment with NAC (10 mM, 30 min) prevented ouabain (*Oua*) (100 nM, 1 h)-stimulated accumulation of the α1 subunit and NHE3 in EE fractions. $n = 4$; **, $p < 0.01$ *versus* control (*Con*), and #, $p < 0.01$ *versus* ouabain treatment. *b,* effect of different concentrations of NAC (30 min) on ouabain (100 nM, 15 min)-induced Tyr(P)$^{418}$ c-Src phosphorylation, $n = 3$; **, $p < 0.01$ *versus* control. *c,* effect of different concentrations of NAC (30 min) on ouabain (100 nM, 1 h)-induced inhibition of active transcellular $^{22}Na^+$ flux in LLC-PK1 monolayer grown on Transwell® membrane support. The transport activity was expressed as relative values from three independent experiments (each performed in triplicate). Sixty minutes after $^{22}Na^+$ (total cpm count was 1,757,980 ± 100,828) was added to the apical compartments, 100 μl of medium from basolateral compartments (total medium volume = 1.0 ml) from each well was collected and counted. $n = 3$, **, $p < 0.01$ and *, $p < 0.05$ *versus* control.

Pretreatment with NAC (10 mM, 30 min) also significantly ($p < 0.01$) attenuated ouabain (100 nM, 1 h)-stimulated accumulation of Na/K-ATPase α1 subunit and NHE3 in EE fractions (Fig. 2*a*). Interestingly, ouabain-induced c-Src activation was significantly ($p < 0.01$) attenuated by pretreatment (30 min) with high doses but not a low dose of NAC in LLC-PK1 cells (Fig. 2*b*). Functionally, ouabain (100 nM, 1 h)-induced inhibition of active transepithelial $^{22}Na^+$ flux was blunted by pretreatment of 10 mM NAC (30 min) but not 1 mM NAC (Fig. 2*c*).

*Ouabain Stimulates Protein Carbonylation*—To evaluate protein oxidation, we used GO-glucose system-induced $H_2O_2$ as a positive control of overall oxidative stress as described previously (5). We first tested whether serum starvation itself affects protein carbonylation. As shown in Fig. 3, *a* and *b*, serum starvation has no effect on basal protein carbonylation level (Fig. 3*a*), and both ouabain (100 nM, 1 h) and GO (3 milliunits/ml, 1 h) significantly ($p < 0.01$) stimulated carbonylation of a broad range of proteins (Fig. 3*b*). Moreover, the carbonylation profile of whole cell lysate is significantly ($p < 0.01$) different from that of EE fractions (Fig. 3*c*), suggesting that ouabain not only stimulated protein carbonylation but also promoted the redistribution of certain carbonylated proteins. When equal amounts of whole cell lysate proteins were derivatized with DNPH and then immunoprecipitated with anti-DNP antibody, both ouabain (100 nM, 1 h) and GO (3 milliunits/ml, 1 h) caused protein carbonylation of the α1 subunit, c-Src and NHE3 (Fig. 3*d*). When whole cell lysate proteins were immunoprecipitated with anti-α1 subunit antibody and then derivatized with DNPH, a similar pattern of the α1 carbonylation stimulated by ouabain and GO occurred (control, 100 ± 5.8.1 *versus* ouabain 261 ± 9.6 *versus* GO 306 ± 10.8, both $p < 0.01$ *versus* control, $n = 3$). Pretreatment with NAC (10 mM, 30 min) significantly ($p < 0.01$) reduced ouabain- and GO-induced protein carbonylation in whole cell lysates (Fig. 3*e*). Pretreatment with (±)-α-tocopherol (Sigma, 100 μM, 30 min) also significantly ($p < 0.05$) attenuated ouabain- and GO-induced protein carbonylation (Fig. 3*f*).

*ROS Directly Inhibits Active Transepithelial $^{22}Na^+$ Flux*—In RPTs, increases in oxidative stress stimulate RPT sodium reabsorption by inhibition of basolateral Na/K-ATPase and apical NHE3 (27–29). We used GO-induced $H_2O_2$ to examine if ouabain-induced ROS affects Na/K-ATPase signaling. Like ouabain, GO (1 and 3 milliunits/ml) activated c-Src (Fig. 4*a*, 15 min of treatment) and stimulated the accumulation of the α1 subunit and NHE3 in EE fractions (Fig. 4*b*, 1 h of treatment, $p < 0.01$). In the active transepithelial $^{22}Na^+$ flux assay, GO (3 milliunits/ml, 1 h) significantly ($p < 0.01$) inhibited the active transepithelial $^{22}Na^+$ flux (Fig. 4*c*). However, ouabain-induced inhibition only occurred when ouabain was applied in the basolateral aspect (Fig. 4*c*). In contrast, GO inhibited transepithelial $^{22}Na^+$ flux in both the basolateral and apical aspects. However, we cannot exclude the possibility that GO-mediated inhibition of transepithelial $^{22}Na^+$ flux is partially due to its effects on other $Na^+$-coupled mechanisms such as the $Na^+$/glucose cotransport system (SGLT) (30). GO-induced c-Src activation and trafficking of the α1 subunit and NHE3 were also significantly ($p < 0.01$) attenuated by pretreatment with NAC (10 mM, 30 min) (Fig. 4, *a* and *b*). NAC alone has no significant effect.

*Role of Na/K-ATPase·c-Src Signaling in Ouabain-induced Protein Carbonylation*—To test the role of the Na/K-ATPase·c-Src signaling complex, we used two stable cell lines generated

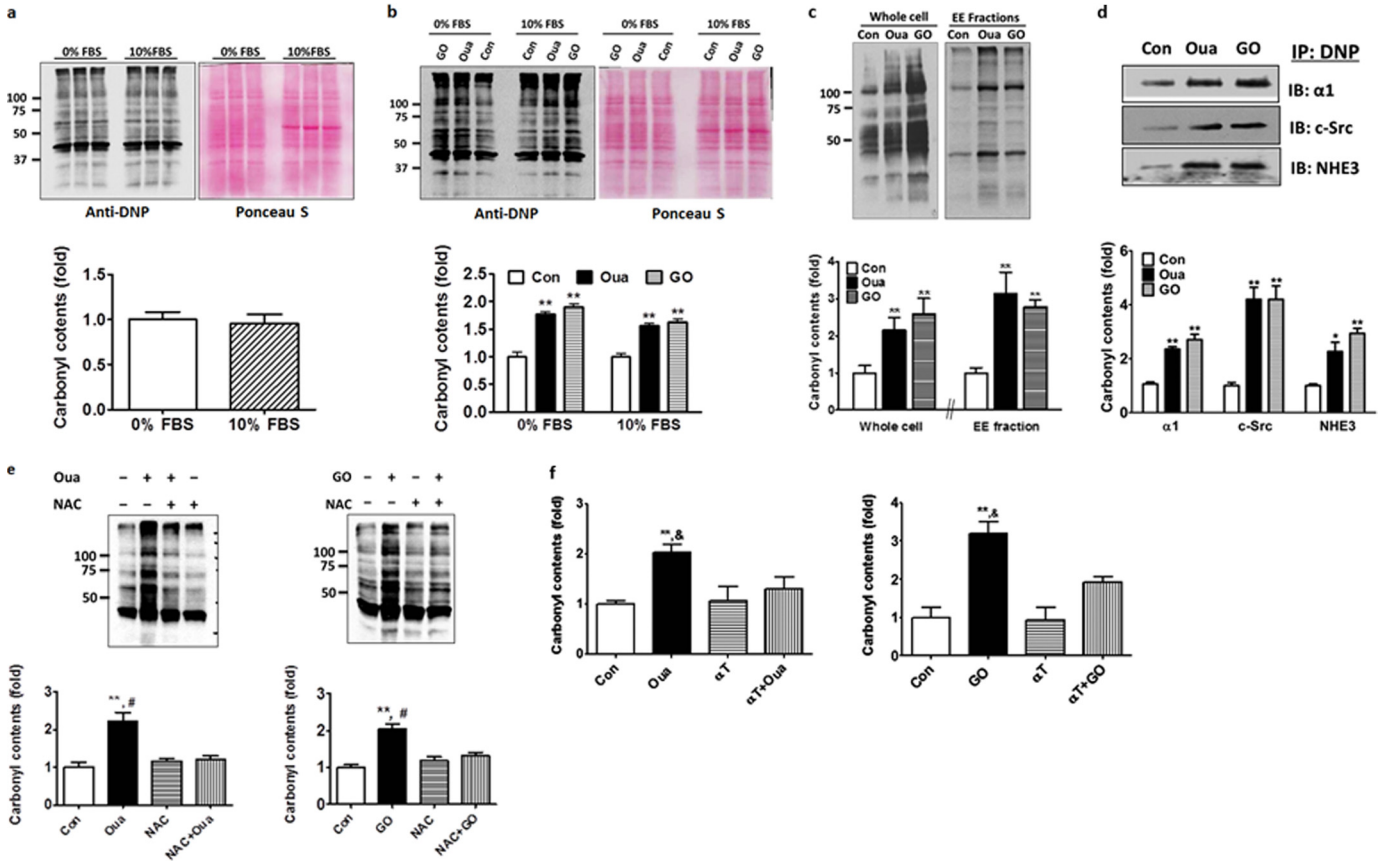



FIGURE 3. **Ouabain stimulates protein carbonylation.** *a,* serum starvation has no significant effect on the basal level of protein carbonylation ($n = 3$). Three serum-starved samples (0% FBS) and three non-serum-starved samples (10% FBS) were used for comparison. Ponceau S staining served as loading control. *b,* both ouabain (*Oua,* 100 nM, 1 h) and glucose oxidase (*GO,* 3 milliunits/ml, 1 h) stimulated protein carbonylation with and without serum starvation ($n = 3$). Ponceau S staining served as loading control (*Con*). *c,* ouabain (100 nM, 1 h) and GO (3 milliunits/ml) stimulated protein carbonylation in both whole cell lysates and EE fractions ($n = 4$). *d,* immunoprecipitation (*IP*) against anti-DNP antibody showed both ouabain- (100 nM, 1 h) and GO (3 milliunits/ml, 1 h)-stimulated protein carbonylation of the α1 subunit, c-Src, and NHE3 ($n = 3$). *e,* pretreatment with NAC (10 mM, 30 min) significantly reduced ouabain (*left panel*), and GO (*right panel*) stimulated protein carbonylation in whole cell lysates ($n = 4$). *f,* pretreatment with (±)-α-tocopherol (*αT*, 100 μM, 30 min) significantly reduced ouabain (*left panel, n* = 5), and GO (*right panel, n* = 3) stimulated protein carbonylation in whole cell lysates. **, $p < 0.01$, and *, $p < 0.05$ *versus* control; #, $p < 0.01$ *versus* pretreatment with NAC plus ouabain or GO; &, $p < 0.05$ *versus* pretreatment with (±)-α-tocopherol plus ouabain or GO. *IB*, immunoblot.

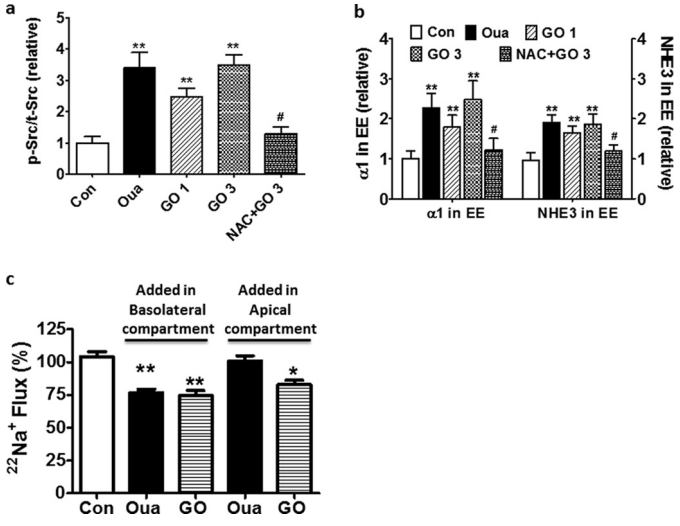



FIGURE 4. **ROS directly inhibits active transepithelial $^{22}Na^+$ flux.** Like ouabain (*Oua*) (100 nM), GO (1 and 3 milliunits/ml) activated c-Src (15 min treatment, $n = 4$) (*a*), accumulated the α1 subunit and NHE3 in EE fractions (1 h treatment, $n = 3$) (*b*), and inhibited active $^{22}Na^+$ flux (1 h treatment, $n = 3$) in LLC-PK1 cells (*c*). **, $p < 0.01$, and *, $p < 0.05$ *versus* control (*Con*). #, $p < 0.01$ *versus* GO (3 milliunits/ml) treatments. NAC (10 mM, 30 min) alone has no significant effects.

from LLC-PK1, α1 knockdown PY-17 cells (express about 8–10% of α1 compared with the parent LLC-PK1) and caveolin-1 knock-out C2-9 cells. Both PY-17 and C2-9 cells have disrupted Na/K-ATPase·c-Src signaling and do not respond to ouabain stimulation in terms of c-Src activation (11, 31, 32). As shown in Fig. 5*a*, ouabain (100 nM, 1 h)-induced protein carbonylation was significantly ($p < 0.01$) attenuated in PY-17 and C2-9 cells.

To examine the role of c-Src kinase, we first used Src kinase (Src, Yes, and Fyn)-null SYF and SYF + c-Src (SYF cells expressing c-Src) cells generated from mouse embryo fibroblasts (33). A higher concentration of ouabain (25 μM) was applied because the mouse α1 subunit is ouabain-resistant. The same GO concentration (3 milliunits/ml) was used because the oxidant sensitivity of the α1 subunit is not dependent on its sensitivity to ouabain. As shown in Fig. 5, *b* and *c*, both ouabain and GO stimulated protein carbonylation in SYF + c-Src cells, which was significantly ($p < 0.01$) attenuated by pretreatment with NAC (10 mM, 30 min). However, in SYF cells, ouabain failed to stimulate protein carbonylation, but GO was still able to stimulate ($p < 0.05$) protein carbonylation, albeit to a lesser degree when compared with SYF + c-Src cells. We further used

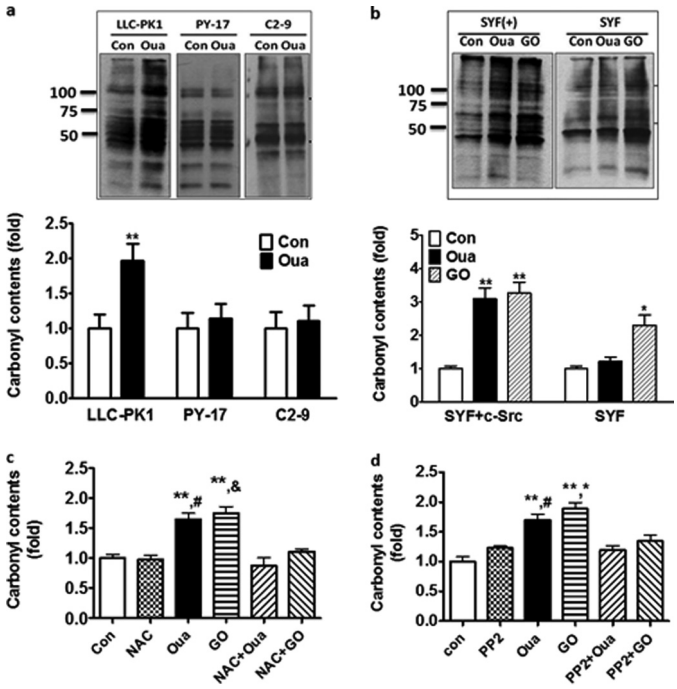



FIGURE 5. **Role of Na/K-ATPase·c-Src signaling complex and c-Src in ouabain- and GO-induced protein carbonylation.** *a,* ouabain (*Oua*) (100 nM, 1 h) stimulated protein carbonylation in whole cell lysate in LLC-PK1 cells but not in the $\alpha$1 subunit knockdown PY-17 cells and caveolin-1 knock-out C2-9 cells. $n = 4$. **, $p < 0.01$ *versus* control (*Con*). *b,* in SYF + c-Src cells, both ouabain (25 $\mu$M, 1 h) and GO (3 milliunits/ml, 1 h) stimulated protein carbonylation in whole cell lysates. In SYF cells, ouabain failed to stimulate protein carbonylation. $n = 4$. **, $p < 0.01$, and *, $p < 0.05$ *versus* control. *c,* in SYF + c-Src cells, pretreatment with NAC (10 mM, 30 min) attenuated ouabain (25 $\mu$M, 1 h) and GO (3 milliunits/ml, 1 h) stimulated protein carbonylation. $n = 4$. **, $p < 0.01$, *versus* control; #, $p < 0.01$ *versus* NAC alone and NAC + ouabain; &, $p < 0.01$ *versus* NAC alone and NAC + GO. *d,* in LLC-PK1 cells, pretreatment with PP2 (10 $\mu$M, 30 min) significantly attenuated ouabain (100 nM, 1 h)- and GO (3 milliunits/ml)-induced protein carbonylation. $n = 3$. **, $p < 0.01$ *versus* contro; #, $p < 0.05$ *versus* PP2 alone and PP2 + ouabain; *, $p < 0.05$ *versus* PP2 alone and PP2 + GO.

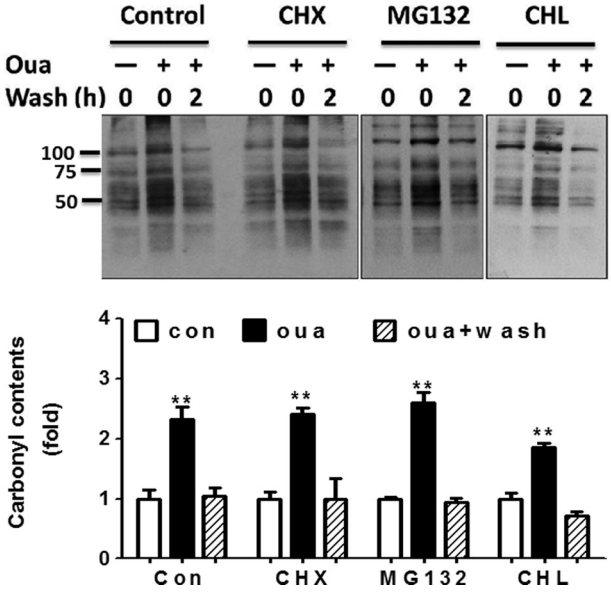



FIGURE 6. **Ouabain-stimulated protein carbonylation is reversible.** For control experiments, LLC-PK1 cells were treated with ouabain (*Oua*) (100 nM for 1 h) to induce protein carbonylation. Control and one set of ouabain-treated cells were collected (wash = 0). In another ouabain-treated set, after ouabain treatment for 1 h, ouabain was removed (by extensive wash with culture medium), and cells were cultured in ouabain-free medium for another 2 h (wash = 2). For inhibitor experiments, LLC-PK1 cells were pretreated for 2 h with cycloheximide (*CHX*, 20 $\mu$g/ml, $n = 5$), MG132 (10 $\mu$M, $n = 3$), or chloroquine (*CHL*, 100 $\mu$M, $n = 4$), followed by the same procedure as above, in the presence of these inhibitors. **, $p < 0.01$ *versus* control (*Con*).

**TABLE 1**

**The spectral counts of $Pro^{222}$/$Pro^{234}$ and $Thr^{224}$/$Thr^{237}$ in two peptides**

Ouabain and GO induced direct carbonylation of $Pro^{222}$ and $Thr^{224}$ in peptide $^{211}$VDNSSLTGESEPQTR$^{225}$. The modified peptide was identified with spectral counts of 0, 3, and 3 in control, ouabain-, and GO-treated samples, respectively.

| | $^{211}$V | D | N | S | S | L | T | G | E | S | E | P | Q | T | $R^{225}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Control** | | | | | | | | | | | | **0** | | **0** | |
| **Ouabain** | | | | | | | | | | | | **3** | | **3** | |
| **GO** | | | | | | | | | | | | **3** | | **3** | |

| | $^{226}$S | P | D | F | T | N | E | N | P | L | E | T | $R^{238}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Control** | | | | | | | | | **2** | | | **2** | |
| **Ouabain** | | | | | | | | | **2** | | | **2** | |
| **GO** | | | | | | | | | **3** | | | **3** | |

the Src kinase inhibitor PP2 to elucidate the role of Src. As shown in Fig. 5*d*, pretreatment with PP2 (10 $\mu$M, 30 min) significantly ($p < 0.01$) attenuated both ouabain- and GO-induced protein carbonylation in LLC-PK1 cells.

*Ouabain-stimulated Protein Carbonylation Is Reversible*—LLC-PK1 cells were treated with ouabain (100 nM, 1 h) to induce protein carbonylation, and then unbound ouabain was removed by extensive washing with culture medium. As shown in Fig. 6, ouabain-induced protein carbonylation was significantly ($p < 0.01$) reduced by removal of ouabain from the culture medium. To test if this is due to *de novo* protein synthesis or degradation, LLC-PK1 cells were pretreated for 2 h with cycloheximide (20 $\mu$g/ml), proteasome inhibitor MG132 (10 $\mu$M), or chloroquine (100 $\mu$M). In comparison with control (no inhibitors), the decreased carbonylation after removal of ouabain was independent of *de novo* protein synthesis and degradation (Fig. 6).

*Ouabain and GO Stimulate Direct Carbonylation in the Na/K-ATPase α1 Subunit*—To determine the carbonylation site(s), the immunoprecipitated $\alpha$1 subunit isolated from LLC-PK1 cells treated either with or without ouabain (100 nM, 1 h) or GO (3 milliunits/ml, 1 h) was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Peptide-to-spectral matching was performed using X!Tandem/TPP software suite considering direct carbonylations on arginine, proline, threonine, and lysine as potential modifications (see under "Experimental Procedures"). This analysis identified $Pro^{222}$, $Thr^{224}$, $Pro^{234}$, and $Thr^{237}$ as direct-carbonylated amino acids on the $\alpha$1 subunit. A tryptic peptide $^{226}$SPDFTNENPLETR$^{238}$ (numbered by UniProtKB/Swiss-Prot No. P05024 (AT1A1_PIG) (precursor ion $[MH]^{2+}$ = 767.36, $\Delta$mass = 20 ppm)) containing carbonylated $Pro^{234}$ and $Thr^{237}$ was observed in all three samples with spectral counts of 2, 2, and 3 in control, ouabain- and GO-treated samples, respectively (Table 1). A modified peptide $^{211}$VDNSSLTGESEPQTR$^{225}$ (precursor ion $[MH]^{+2}$ = 817.38, $\Delta$mass = 13 ppm) containing carbonylated $Pro^{222}$ and $Thr^{224}$ was observed only in peptide in ouabain- and GO-treated cells with spectral counts of 0, 3 and 3 in control, ouabain and GO treated samples, respectively (Table

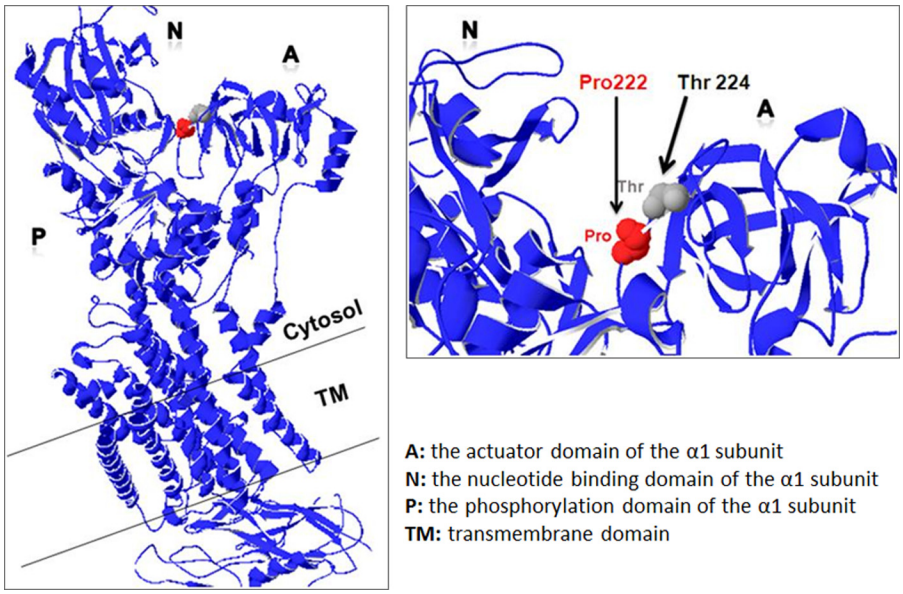



FIGURE 7. **Ouabain and GO stimulates direct carbonylation of the Na/K-ATPase α1 subunit.** A ribbon diagram of the Na/K-ATPase α1 subunit with $Pro^{222}$ and $Thr^{224}$ highlighted (Protein Data Bank code 2ZXE).

1). $Pro^{222}$ and $Thr^{224}$ are located on the surface of the actuator (A) domain of the α1 subunit, facing the nucleotide binding (N) domain (Fig. 7, *ribbon diagram*). Moreover, these two peptides ($^{211}$VDNSSLTGESEPQTRSPDFTNENPLETR$^{238}$) are contiguous in the primary sequence and are located in the A domain of the α1 subunit.

## DISCUSSION

Recent studies have demonstrated the important role of endogenous CTS in the regulation of renal sodium excretion and blood pressure (34–36). These include transgenic mice with a "humanized" ouabain-sensitive Na/K-ATPase α1 subunit, CTS infusion, and immunoneutralization of endogenous CTS (12, 22, 37–40). For many years, the concept of CTS elimination of excessive sodium by direct inhibition of Na/K-ATPase has been a topic of debate. The newly appreciated signaling function of Na/K-ATPase has been widely confirmed and has provided a novel mechanistic framework. We have demonstrated that activation of this Na/K-ATPase signaling function inhibits RPT sodium reabsorption to correct sodium retention-related volume expansion and blood pressure increase. We conclude that, rather than contributing to development and maintenance of hypertension, properly regulated RPT Na/K-ATPase signaling has a protective effect under physiological settings (12, 13, 21, 22). However, a fundamental unanswered question is the underlying mechanism to "turn on/off" the RPT Na/K-ATPase signaling. Marinobufagenin, another ligand of the Na/K-ATPase, stimulates ROS generation, protein oxidation, trafficking of RPT Na/K-ATPase, and urinary sodium excretion in experimental animals (12, 40, 41). The impaired RPT Na/K-ATPase·c-Src signaling in Dahl salt-sensitive rats (22) prompted us to investigate the underlying mechanisms. We report here that, in LLC-PK1 cells, ROS and ouabain-Na/K-ATPase·c-Src signaling are inextricably linked. ROS is a critical signaling mediator of ouabain-stimulated RPT Na/K-ATPase·c-Src signal transduction. Specifically, carbonylation of the α1 subunit is involved in a feed-forward mechanism in the regulation of Na/K-ATPase signal transduction and subsequent inhibition of transepithelial $^{22}Na^+$ flux. The present data further indicate that the carbonylation modification of the α1 subunit is a key mechanism in the ouabain-stimulated regulation of RPT Na/K-ATPase signaling and sodium handling. Moreover, because GO alone inhibited transepithelial $^{22}Na^+$ flux and stimulated NHE3 carbonylation and redistribution, ouabain-induced ROS generation and protein carbonylation may function as the link from ouabain-Na/K-ATPase signaling to NHE3 regulation. Because oxidative stress inhibits other $Na^+$-coupled mechanisms such as the $Na^+$/glucose cotransport system (SGLT) (30), we cannot exclude the possibility that ouabain- and GO-mediated inhibition of transepithelial $^{22}Na^+$ flux might partially be due to their effects on other $Na^+$-coupled mechanisms. Moreover, even though ouabain stimulated the superoxide-related ROS generation, we also cannot exclude the possibility that the oxidative effect of ouabain might involve other pathways or mechanisms.

We used the GO-glucose system to mimic the overall oxidative stress and used NAC as a scavenger of $H_2O_2$ and protein carbonylation. GO (3 milliunits/ml) induces a low and sustained generation of $H_2O_2$ (3–5) that can stimulate Src kinase tyrosine phosphorylation (42) and Na/K-ATPase endocytosis (5). NAC is one of the most bioavailable precursors of the reducing agent glutathione (43) and is more effective in reducing direct protein carbonylation (44, 45) than traditional reactive carbonyl species scavengers (46). We have shown that pretreatment with NAC (10 mM, 30 min) prevents ouabain-stimulated ROS generation and Na/K-ATPase signaling (3, 4). Pretreatment with NAC attenuated the effects induced by ouabain, including c-Src activation, protein carbonylation, and protein trafficking. Pretreatment with (±)-α-tocopherol also attenuated ouabain- and GO-induced protein carbonylation. Functionally, pretreatment with NAC either partially or com-

pletely prevents ouabain-induced inhibition of transepithelial $^{22}Na^+$ flux in a dose-dependent manner, demonstrating that the ouabain-Na/K-ATPase signaling axis requires the presence of a basal physiological ROS level. We have reported that NAC (10 mM) inhibited ouabain (100 $\mu$M, approximate estimated $IC_{50}$ in terms of inhibition of $^{86}Rb^+$ uptake)-induced ROS generation, but did not prevent ouabain (10 min)-induced inhibition of the initial rate of $^{86}Rb^+$ uptake, a measurement of Na/K-ATPase ion exchange activity, in rat neonatal cardiac myocytes (3). It is important to note that we have not carefully examined the long term effects of low doses of ouabain on the surface expression of Na/K-ATPase and the pumping activity of Na/K-ATPase in rat neonatal cardiac myocytes. Rat neonatal cardiac myocytes express ouabain-resistant Na/K-ATPase $\alpha$1 and ouabain-sensitive $\alpha$3 subunits, although LLC-PK1 cells predominantly express the ouabain-sensitive $\alpha$1 subunit. In LLC-PK1 cells, a low concentration of ouabain (up to 100 nM, about 1/10th of $IC_{50}$) does not inhibit $^{86}Rb^+$ uptake nor alter intracellular $Na^+$ concentration when administered for up to 15 min (13, 47), but it does inhibit $^{86}Rb^+$ and $Na^+$ uptake when administered for longer periods as we reported previously (13, 47, 48), due to ouabain-induced Na/K-ATPase endocytosis via Na/K-ATPase signaling (11, 13, 21, 47, 48). In LLC-PK1 cells, ouabain (1 h)-induced inhibition of transepithelial $^{22}Na^+$ flux is mostly dependent on the coordinated regulation of Na/K-ATPase and NHE3 through Na/K-ATPase signaling (13, 21, 47). Ouabain induced redistribution of Na/K-ATPase and NHE3 in LLC-PK1 cells, with a resultant reduction in cell surface levels of both transporters to depress apical $Na^+$ entry through NHE3 (and other $Na^+$-coupled $Na^+$ transporters) and basolateral $Na^+$ extrusion through Na/K-ATPase. Ouabain-induced redistribution of Na/K-ATPase and NHE3 and inhibition of $^{22}Na^+$ flux were inhibited by c-Src or PI3K inhibitors, indicating the involvement of Na/K-ATPase signaling which, as we show, is sensitive to NAC pretreatment. This strengthens our hypothesis that ouabain induces ROS generation largely through Na/K-ATPase signaling, and ROS regulates Na/K-ATPase signaling and function.

Although disruption of the Na/K-ATPase·c-Src signaling (as in PY-17 and C2-9 cells) attenuated ouabain-stimulated protein carbonylation, studies with SYF/SYF + c-Src cells and Src kinase inhibitor PP2 are consistent with our previous observations that ouabain stimulates a c-Src-dependent regulation of RPT Na/K-ATPase and NHE3 (13, 21). Interestingly, GO-stimulated protein carbonylation was also attenuated in SYF cells, suggesting that the Na/K-ATPase·c-Src signaling complex is capable of functioning as a receptor complex for extracellular $H_2O_2$.

Ouabain-stimulated protein carbonylation was reversed after removal of unbound ouabain from the culture medium. Direct protein carbonylation mostly occurs on lysine, arginine, threonine, and proline residues. These carbonylation modifications, via metal-catalyzed activation of $H_2O_2$, are very stable and chemically irreversible. Oxidatively damaged molecules need to be tightly regulated, either by reduction through a reducing process or degradation and replacement by a newly synthesized protein. A recent study demonstrated the role of the carbonylation/decarbonylation process in signal transduction in which thiol groups were responsible for decarbonylation via enzymatic processes probably through thioredoxin reductase (49). Our present data suggest an undefined self-decarbonylation mechanism to reverse the carbonylation. This decarbonylation process was independent of both *de novo* protein synthesis and degradation through lysosome and proteasome pathways, leading us to speculate a likely enzyme-driven mechanism of the removal of the carbonyl group, by either a known enzyme system or an unidentified enzyme-like protein. Nevertheless, the underlying mechanism might be physiologically significant because the carbonylation/decarbonylation process could be an important regulator of the RPT Na/K-ATPase signaling and sodium handling.

Upon ouabain binding, Na/K-ATPase undergoes a conformational change in which the A domain rotates toward the N domain by using the $^{217}$TGES$^{220}$ motif as the anchor and the transmembrane M1/M2 domain shifted toward the M3/M4 domain (50). Structure-function analysis indicates that this conformational change might affect protein/protein interactions between the $\alpha$1 subunit and its signaling partners such as c-Src, PI3K, and inositol 1,4,5-trisphosphate receptor (50), which are critical in Na/K-ATPase signaling. To the best of our knowledge, this is the first study to demonstrate that CTS causes a direct and reversible carbonylation of the $\alpha$1 subunit A domain, which can significantly regulate Na/K-ATPase signaling and transepithelial sodium transport.

Our present data demonstrate that ROS may be a critical mediator of RPT Na/K-ATPase signaling and sodium reabsorption. Future studies will be necessary to clarify how carbonylation modification of the $\alpha$1 subunit is able to alter RPT Na/K-ATPase signaling and sodium handling.

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