Protein Carbonylation of an Amino Acid Residue of the Na/K-ATPase α1 Subunit Determines Na/K-ATPase Signaling and Sodium Transport in Renal Proximal Tubular Cells

Yanling Yan, PhD; Anna P. Shapiro, MD; Brahma R. Mopidevi, PhD; Muhammad A. Chaudhry, PhD; Kyle Maxwell, BSc; Steven T. Haller, PhD; Christopher A. Drummond, PhD; David J. Kennedy, PhD; Jiang Tian, PhD; Deepak Malhotra, MD, PhD; Zi-jian Xie, PhD; Joseph I. Shapiro, MD; Jiang Liu, MD, PhD

Background—We have demonstrated that cardiotonic steroids, such as ouabain, signaling through the Na/K-ATPase, regulate sodium reabsorption in the renal proximal tubule. By direct carbonylation modification of the Pro222 residue in the actuator (A) domain of pig Na/K-ATPase α1 subunit, reactive oxygen species are required for ouabain-stimulated Na/K-ATPase/c-Src signaling and subsequent regulation of active transepithelial $^{22}$Na$^+$ transport. In the present study we sought to determine the functional role of Pro222 carbonylation in Na/K-ATPase signaling and sodium handling.

Methods and Results—Stable pig α1 knockdown LLC-PK1-originated PY-17 cells were rescued by expressing wild-type rat α1 and rat α1 with a single mutation of Pro224 (corresponding to pig Pro222) to Ala. This mutation does not affect ouabain-induced inhibition of Na/K-ATPase activity, but abolishes the effects of ouabain on Na/K-ATPase/c-Src signaling, protein carbonylation, Na/K-ATPase endocytosis, and active transepithelial $^{22}$Na$^+$ transport.


Key Words: Na/K-ATPase • protein carbonylation • protein trafficking • reactive oxygen species • signaling • sodium transport

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inding of ouabain, one of the cardiotonic steroids, to the Na/K-ATPase α1 subunit stimulates multiple protein kinase signaling cascades. One of the downstream effects of ouabain-stimulated Na/K-ATPase signaling is Ras-dependent superoxide-related reactive oxygen species (ROS) generation, which is an integrated component of ouabain-mediated Na/K-ATPase signaling.Ⅰ,Ⅱ We have reported that cardiotonic steroids stimulate ROS generation in different in vitro and in vivo models and also that increases in H$_2$O$_2$ activate Na/K-ATPase signaling pathways and promote Na/K-ATPase endocytosis.Ⅰ⁻Ⅷ

The effect of ROS on the Na/K-ATPase activity has been well documented.Ⅸ⁻Ⅻ Oxidative modification, such as glutathionylation of cysteine residue(s) of the Na/K-ATPase α1 subunit Ⅺ and β1 subunit,Ⅹ inhibits the Na/K-ATPase activity by either stabilizing the enzyme in an E2-prone conformation or by blocking the ATP-binding site. We have reported that while ouabain stimulates ROS generation via the Ras/Rac cascade of the Na/K-ATPase signaling pathways, increases in ROS also activate the Na/K-ATPase signaling, allowing the formation of a Na/K-ATPase/c-Src/Ros signaling amplification loop.Ⅰ⁻Ⅴ,Ⅶ,Ⅷ,Ⅾ Recently, we have further demonstrated that ROS causes direct protein carbonylation of Pro222 of the actuator (A) domain of the α1 subunit of Na/K-ATPase in pig LLC-PK1 cells.Ⅵ This carbonylation modification process is dependent on c-Src activation as demonstrated by using the Src-deficient SYF and c-Src reconstituted SYF+c-Src cells.Ⅵ Functionally, ouabain and increases in H$_2$O$_2$ stimulate Na/K-ATPase signaling, protein carbonylation, and redistribution of Na/K-ATPase and NHE3, leading to the inhibition of active transepithelial $^{22}$Na$^+$ flux.Ⅵ
Our in vitro data suggest that protein carbonylation of Na/K-ATPase α1 subunit may be a novel regulatory mechanism of Na/K-ATPase signaling. However, the functional role of Pro224 carbonylation in the Na/K-ATPase signaling is unclear. We report here that mutation of Pro224 (as Pro222 in pig α1) to Ala in the rat α1 subunit does not affect ouabain-induced inhibition of the Na/K-ATPase activity, but the mutation abolishes ouabain-induced Na/K-ATPase signaling, protein carbonylation, endocytosis of the Na/K-ATPase, and inhibition of active transepithelial 22Na⁺ transport. Taken together, we suggest that Pro224 of rat α1 dictates the renal proximal tubule (RPT) Na/K-ATPase signaling and sodium transport, and that carbonylation modification of Pro224 functions as a signaling amplifier of Na/K-ATPase signaling.

Materials and Methods

Chemicals and Antibodies

All chemicals, except otherwise mentioned, were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies against Na/K-ATPase α1 subunit (clone α6F and clone C464.6) and β1 (clone C464.8) were from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA) and EMD Millipore Upstate (Billerica, MA), respectively. Monoclonal anti-rat IA) and EMD Millipore Upstate (Billerica, MA), respectively. Monoclonal anti-rat IA) and EMD Millipore Upstate (Billerica, MA), respectively. Monoclonal antibodies against total c-Src and tyrosine phosphorylation (p-Tyr, clone PY99) were from Santa Cruz Sciences Center, Lubbock, TX. Polyclonal antibody against 2,4-dinitrophenyl hydrazone derivatives were from Sigma-Aldrich. Radioactive [3H]-ouabain, 86RbCl, and 22NaCl were from Perkin Elmer (Shelton, CA).

Cell Cultures

Cells were cultured with Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a 5% CO₂ humidified incubator. Culture medium was changed daily until confluence. Cells were serum-starved for 16 to 18 hours before treatment. In assays for active transepithelial 22Na⁺ 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. The transepidermal electrical resistance was measured with EVOM2 Epithelial Voltomhmemeter system (World Precision Instruments, Sarasota, FL).

Generation of Mutant Cells

For the present study, neither a protocol approved by Institutional Review Board nor a protocol approved by institutional Animal Care and Use Committee was required. The wild-type AAC-19 cells as well as mutant P224A and A416P cells were all generated from PY-17 cells by using pRc/CMV1-rat α1 plasmid that was also kindly provided by Dr Pressley. The PY-17 is a stable cell line, generated from pig LLC-PK1 cells, with knockdown of pig α1 by siRNA method. The PY-17 cells only expressed ≈8% to 10% of pig α1 compared to parent LLC-PK1 cells. The pRc/CMV1-rat α1 plasmid has been used to develop several stable cell lines including rat α1 rescued AAC-19 (expressing full-length wild-type rat α1 14,15) and A416P cells (expressing Ala416/Pro416 mutation in rat α1 16). The mutant P224A stable cell line (expressing Pro224/Ala224 mutation in rat α1) was generated in the same way as generation of A416P cells. The LLC-PK1 cells were used to evaluate the total α1 expression level, and the PY-17 cells were used to evaluate the expression level of endogenous pig α1 in AAC-19, P224A, and A416P cells.

The Pro222 of pig α1 (UniProtKB/Swiss-Prot No P05024) in LLC-PK1 cells corresponds to the Pro224 of the rat α1 (No P06685, Table 1). Site-directed mutagenesis was used to convert Pro224 of rat α1 to generate single Pro224/Ala224 mutation with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). The mutated sequence was confirmed by DNA sequencing. To develop stable cell lines, transfected ouabain-resistant colonies were selected with ouabain (3 μmol/L) for 1 week post-transfection to eliminate endogenous ouabain-sensitive pig α1 as well as untransfected PY-17 cells because pig α1 subunit is highly sensitive to ouabain compared to rat α1.1,7 Survived colonies expressing rat α1 mutants were expanded into single-cell stable cell lines and verified by specific anti-rat α1 antibody (anti-NASE) and [3H] ouabain binding assay. Wild-type rat α1-rescued PY-17 cell, AAC-19, was used for control in the following studies.

Table 1. Partial Alignment of the α1 Subunit

| SP/P05023 | AT1A1_HUMAN | 181 CKVDNSSLTGESQ222QTRSPDFTNENPLETR 240 |
| SP/P05024 | AT1A1_PIG   | 179 CKVDNSSLTGESQ222QTRSPDFTNENPLETR 238 |
| SP/P06685 | AT1A1_RAT   | 181 CKVDNSSLTGESQ222QTRSPDFTNENPLETR 240 |
| SP/Q8VDN2 | AT1A1_MOUSE | 181 CKVDNSSLTGESQ222QTRSPDFTNENPLETR 240 |

The sequences were obtained from UniProtKB/Swiss-Prot.
Isolation of Early Endosome (EE) Fractions

The EE fractions were isolated by sucrose flotation centrifugation, and the enrichment of EE fractions was verified by the EE marker Rab5 as we previously described. An equal amount of total protein from each sample was precipitated with trichloroacetic acid for Western blot analysis of Na/K-ATPase α1 and β1 subunits. After immunoblotting with the α1 and β1 subunits, the same membrane was stripped and immunoblotted for Rab5 to serve as loading control. Endocytosed α1 and β1 subunits were normalized by Rab5.


To evaluate the cell surface expression level of endogenous pig Na/K-ATPase α1 subunit,[^3]H-ouabain binding assay was performed as described before. The[^3]H-ouabain binding was calibrated with protein content and expressed as the percentage of pig α1 knockdown PY-17 cells. Each experiment was performed in triplicate.

Enzymatic and Ion-Exchange Activity Assays

The enzymatic activity of the Na/K-ATPase was performed by using BIOMOL GREEN Reagent (Enzo Life Science) as described in . Briefly, cells were homogenized, briefly sonicated, and centrifuged (800g for 10 minutes) in ice-cold buffer A (150 mmol/L sucrose, 5 mmol/L HEPES, 4 mmol/L EGTA, 0.8 mmol/L dithiothreitol, pH 7.4). The crude membrane pellet was obtained after centrifugation of the postnuclear fraction (45 000g for 45 minutes) and was resuspended in buffer A to determine protein concentration. The crude membrane samples were treated with alamethicin (0.1 mg/mg of protein) for 10 minutes at room temperature and then added to the buffer B (50 mmol/L Tris, 1 mmol/L EGTA, 1 mmol/L MgCl2, 25 mmol/L KCl, 100 mmol/L NaCl, 5 mmol/L Na2S, pH 7.4). After 15 minutes of pre-incubation at 37°C, the reaction was started by adding ATP/Mg2+ (final concentration of 2 mmol/L) and continued for 45 minutes, and then stopped by adding 8% ice-cold trichloroacetic acid. Phosphate generated during the ATP hydrolysis was measured by BIOMOL GREEN Reagent. Ouabain-sensitive Na/K-ATPase activities were calculated as the difference between the presence and absence of 1 mmol/L ouabain.

To evaluate the transport activity of the Na/K-ATPase and NHE3, 86Rb+ and H+-driven 22Na+ uptake were performed as previously described. Prior to the initiation of the 86Rb+ uptake assay, cellular Na+ was “clamped” with 20 μmol/L monensin for 15 minutes to assure the measurement of the maximal capacity of total active 86Rb+ uptake and to minimize the potential effect of changes in intracellular Na+. The assay was stopped 10 minutes after adding 86Rb+ (≈1 μCi/mL medium) by washing 3 times with ice-cold 100 mmol/L MgCl2 solution. In parallel, ouabain-insensitive 86Rb+ uptake (pretreated with 2 mmol/L ouabain for 15 minutes) was measured in the presence of monensin. Ouabain-sensitive 86Rb+ uptake was calculated by subtraction of ouabain-insensitive 86Rb+ uptake from total 86Rb+ uptake. Prior to H+-driven 22Na+ uptake assay, cells were pretreated with 50 μmol/L amiloride for 30 minutes to inhibit amiloride-sensitive NHE1 activity without significant inhibition of NHE3 and Na/K-ATPase. This allows the measurement of acid-stimulated Na+ entry mainly mediated through apical NHE3. To determine H+-driven 22Na+ uptake, after treated with or without ouabain (10 μmol/L, 1 hour), cells were first rinsed 3 times with Na+-free buffer (in mmol/L, N-methyl-D-glucamine [NMDG+] Cl 140, KCl 4, MgCl2 2, CaCl2 1, and HEPES 10, pH 7.4) and acid loaded for 10 minutes in ammonium-containing Na+-free buffer in which 30 mmol/L NMDG+ was replaced with 30 mmol/L NH4Cl (in mmol/L, NMDGGlCl 110, NH4Cl 30, KCl 4, MgCl2 2, CaCl2 1, and HEPES 10, pH 7.4). The 22Na+ uptake was initiated by replacing the NH4+-containing buffer with Na+-free buffer containing 2 mmol/L 22NaCl (≈1 μCi/mL buffer). The 22Na+ uptake was stopped after 4 minutes by washing 4 times with ice-cold saline. Each experiment was performed in triplicate.

To determine the effect of ouabain (10 μmol/L, 1 hour) on Na/K-ATPase transport capacity, cells were pretreated with or without ouabain (10 μmol/L, 1 hour) prior to the assays. Since rat α1 subunit is ouabain-resistant compared to pig α1 subunit, we chose 10 μmol/L of ouabain that is able to stimulate Na/K-ATPase/c-Src signaling in rat RPT primary cultures.

Active Transepithelial 22Na+ Flux Assay

Cells were cultured on Transwell membrane support to form monolayers and pretreated with 50 μmol/L amiloride for 30 minutes to inhibit amiloride-sensitive NHE1 activity. Active transepithelial 22Na+ flux (from apical to basolateral compartment) was determined by counting radioactivity in the basolateral aspect 1 hour after 22Na+ addition to the apical compartment as previously described. Each experiment was performed in triplicate.

To determine the effect of ouabain (10 μmol/L, 1 hour) on transepithelial 22Na+ transport capacity, cells were pretreated with or without ouabain (10 μmol/L, 1 hour) in basolateral compartments prior to the 22Na+ flux assay.

Assessment of Phosphorylation of Tyrosine, c-Src, and ERK1/2

Cells were treated with and without ouabain (10 μmol/L, 1 hour). Whole cell lysates were prepared with Nonidet P-40

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buffer (containing 1% Nonidet P-40, 0.25% sodium deoxy-
cholate, 50 mmol/L NaCl, 50 mmol/L HEPES, 10% glycerol
[pH 7.4], 1 mmol/L sodium vanadate, 0.5 mmol/L sodium
fluoride, 1 mmol/L phenylmethanesulfonyl fluoride, and pro-
tease inhibitor cocktail for general use [Sigma-Aldrich]).
Phosphorylation was determined with anti-phospho-tyrosine
(p-Tyr) antibody, anti-Src (pY418), and anti-ERK1/2 phospho-
specific antibodies. For tyrosine phosphorylation assessment,
after immunoblotting for tyrosine phosphorylation, the same
membrane was stripped and immunoblotted for actin to serve
as loading control. For c-Src phosphorylation assessment,
after immunoblotting for phospho-c-Src (p-Src), the same
membrane was stripped and immunoblotted for total c-Src
(t-Src). Activation of c-Src was expressed as the ratio of p-Src/t-Src with both measurements normalized to 1.0 for
the control samples. The assessment of ERK1/2 phosphorylation
was performed in the same way as described for c-Src.

**Assessment of Protein Carbonylation**

Protein carbonylation was determined by Western blot
analysis as we described before. Briefly, an equal amount
of total protein from each sample was denatured with 6% SDS
(final concentration), derivatized with 2,4-dinitrophenylhydra-
zeine (freshly prepared, 10 mmol/L in 1 N HCl) to form
2,4-dinitrophenyl hydrazone derivatives, and then neutralized
with neutralization buffer (30% of glycerol in 2 mol/L Tris).
This was followed by Western blot for protein carbonylation
assay. The signal density values of control samples were
normalized to 1.0 with Ponceau S staining as loading control.

**Western Blotting**

For Western blot analysis, equal amounts of total protein were
resolved by 10% SDS-PAGE, transferred onto a polyvinylidene
difluoride membrane (EMD Millipore), and immunoblotted with
indicated antibodies. Signal detection was performed with an
enhanced chemiluminescence SuperSignal kit (Pierce, Rock-
ford, IL). Multiple exposures were analyzed to assure that the
signals were within the linear range of the film. The signal
density was determined using NIH ImageJ 1.48v software.

**Bioinformatics Analysis of the Pro222 of the Na/
K-ATPase**

DeepView-Swiss-PdbViewer (v4.1) integrated with SWISS-
MODEL via the ExPASy web server, a fully automated protein
structure homology-modeling server used to generate a
3-dimensional structure of a protein from its amino acid
sequence. Modeling of pig Na/K-ATPase E1P and E2P
structures was based on 3WGU and 4RES pdb structures,
respectively. The pig c-Src kinase crystal structure was
derived from pdb 1Y57. The rat Na/K-ATPase E1P and E2P
structures were derived from 3WGU and 4RES. The alignment
of pig and rat Na/K-ATPase α1 subunit showed over 98% sequence
similarity. The quality of structures was further assessed by using Structure Analysis and Verification Server
(v4). The protein–protein docking analysis was performed by
using Z-DOCK web server and the protein–protein interac-
tion was visualized by using Accelrys Discovery Studio
Visualizer v2.5.5.

**Statistical Analysis**

Data were tested for normality (by SPSS Shapiro–Wilk
normality test) and then subjected to parametric analysis.
When more than 2 groups were compared, 1-way ANOVA 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 <0.01 levels. SPSS software was used for
all analysis. Values are given as mean±SEM.

**Results**

**Generation of Stable P224A Mutant Cells**

As shown in Figure 1, P224A mutant cells expressed a slightly
higher but relatively comparable level of mutated rat α1 in
comparison with AAC-19 cells. The expression of rat α1 was
confirmed by a rat α1-specific antibody (anti-NASE), and the
total α1 (both endogenous pig α1 and rat α1) with a generic α1-
specific antibody α6F (Figure 1A). The expression of mutated
rat α1 in P224A mutant cells was predominantly located on the

[rest of the text continues]
stimulation in terms of c-Src activation, suggesting that the remaining endogenous pig α1 would not interfere with ouabain-mediated signaling and function originated from rat α1 and β1 subunits. Functionally, the mutant P224A and A416P cells showed a similar sensitivity to ouabain as AAC-19 cells, in terms of ouabain-sensitive enzymatic activity of the Na/K-ATPase in crude membrane preparations (Figure 1D) as well as the ion-exchange activity assayed by ouabain-sensitive 86Rb+ uptake (Figure 1E). The data indicate that P224A mutation did not change the characteristics of ouabain-induced Na/K-ATPase inhibition.

Ouabain-Stimulated Protein Tyrosine Phosphorylation

In a different type of cells, ouabain stimulated tyrosine phosphorylation of multiple proteins in a c-Src-dependent manner that is crucial in ouabain-stimulated Na/K-ATPase signaling. In contrast to c-Src reconstituted SYF+c-Src cells, depletion of Src family kinases (Ssrc, Yes, and Fyn) prevented ouabain (10–100 μmol/L)-induced protein tyrosine phosphorylation and Na/K-ATPase endocytosis in SYF cells. As shown in Figure 2, ouabain (10 μmol/L, 1 hour) significantly stimulated tyrosine phosphorylation of multiple proteins in AAC-19 (P<0.01) and A416P (P<0.01), but not in P224A cells, suggesting that P224A mutation might prevent ouabain-mediated Na/K-ATPase signaling.

Ouabain-Stimulated Protein Carbonylation and Na/K-ATPase Signaling

As shown in Figure 3, ouabain (10 μmol/L, 1 hour) significantly stimulated carbonylation of a broad range of proteins in AAC-19 (P<0.01) and A416P (P<0.01), but not in P224A cells (Figure 3A). The P224A mutation significantly (P<0.01) attenuated ouabain (10 μmol/L, 1 hour)-stimulated c-Src and ERK1/2 activation as seen in AAC-19 and A416P mutant cells (Figure 3B and 3C). The ouabain-induced effects in AAC-19 and A416P cells were similar to those seen in LLC-PK1 cells. These data indicate that Pro224 of the rat α1 subunit might be essential in ouabain-stimulated protein carbonylation and Na/K-ATPase signaling.
Ouabain-Induced Endocytosis of Na/K-ATPase

As shown in Figure 4, in AAC-19 cells, both ouabain (10 μmol/L, 1 hour) and glucose oxidase (GO, 3 mU/mL, 1 hour) stimulated accumulation of Na/K-ATPase α1/β1 subunits in EE fractions (P<0.01), which is consistent with our previous observations in LLC-PK1 cells and rat RPTs. However, ouabain-induced endocytosis of Na/K-ATPase was significantly attenuated (P<0.01) with the P224A mutation. Functionally, the data are consistent with the observation that ouabain (10 μmol/L, 1 hour)-induced inhibition of active transepithelial 22Na+ flux was blunted by the P224A mutation (Table 2). It is worth noting that both ouabain- and glucose oxidase–stimulated Na/K-ATPase endocytosis was prevented by the P224A mutation, consistent with our previous observation in Src kinase null SYF cells.

Ouabain-Induced Reduction of Transepithelial 22Na+ Transport

In pig LLC-PK1 cells and rat proximal tubular cells, ouabain treatment stimulated internalization of the Na/K-ATPase and NHE3 via the Na/K-ATPase signaling. This led to the reduction
of the Na/K-ATPase and NHE3 on the cell surface, which further led to reduced Na⁺ entry (mainly mediated by NHE3) and Na⁺ extrusion (mainly mediated by the Na/K-ATPase) and thus reduced active transepithelial ²²Na⁺ transport of the cells.¹⁸,¹⁹,²³,³⁰–³² To evaluate the effect of the mutation of P224A and A416P on Na/K-ATPase-mediated Na⁺ extrusion (by ⁸⁶Rb⁺ uptake assay), NHE3-mediated Na⁺ entry (by H⁺-driven ²²Na⁺ uptake assay) and active transepithelial ²²Na⁺ transport (by active transepithelial ²²Na⁺ flux assay), AAC-19, P224A, and A416P cells were treated with or without ouabain (10⁻⁹ mol/L, 1 hour) and then the assays were performed. As shown in Table 2, ouabain significantly inhibited the cellular Na⁺ entry and extrusion as well as transepithelial ²²Na⁺ transport in AAC-19 and A416P cells, but not in P224A cells. The data indicate that, while ouabain was able to stimulate similar functional changes in AAC-19 and A416P cells, as seen in LLC-PK1 and renal RPTs,¹⁸,²³ the P224A mutation prevented ouabain-mediated regulation. However, we could not exclude the possible effect of chloride-couple cation carriers and K⁺ channels.

### Bioinformatics Analysis of the Pro222 of the Na/K-ATPase

The bioinformatics analysis indicated that pig Pro222 carbonylation and Ala222 in Pro/Ala mutation did not affect

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**Figure 4.** P224A mutation prevents ouabain-induced Na/K-ATPase endocytosis. P224A mutation prevents ouabain (10 µmol/L, 1 hour)-stimulated accumulation of α1 and β1 subunit in early endosome fractions. A representative Western blot and quantitative analysis were shown. n=4, **P<0.01 vs control AAC-19 cells.

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**Table 2.** Ouabain (10 µmol/L, 1 Hour)-Inhibited Activities of Na/K-ATPase and NHE3 as Well as Active Transepithelial ²²Na⁺ Flux in AAC-19 and A416P Cells, but Not in P224A Mutant Cells

<table>
<thead>
<tr>
<th></th>
<th>AAC-19</th>
<th>P224A</th>
<th>A416P</th>
<th>Ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>⁸⁶Rb⁺ uptake</td>
<td>100±6.1</td>
<td>76.4±5.5**</td>
<td>100±3.1</td>
<td>101.9±3.6</td>
</tr>
<tr>
<td>H⁺-driven ²²Na⁺ uptake</td>
<td>100±4.6</td>
<td>69.7±3.7**</td>
<td>100±5.3</td>
<td>101.0±3.1</td>
</tr>
<tr>
<td>Transepithelial ²²Na⁺ flux</td>
<td>100±4.7</td>
<td>72.5±5.5**</td>
<td>100±6.7</td>
<td>98.1±3.4</td>
</tr>
</tbody>
</table>

After treatment with or without ouabain (10 µmol/L, 1 hour), assays were performed as described in “Enzymatic and Ion-Exchange Activity Assays” and “Active Transepithelial ²²Na⁺ Flux Assay” under “Experimental Methods.” For ouabain-sensitive ⁸⁶Rb⁺ uptake assay, the control values are (in CPM/100 µg protein) AAC-19, 10 913.9±1356; P224A, 14 975.7±2149; and A416P, 13 426.9±1440. For H⁺-driven ²²Na⁺ uptake assay, the control values are (in CPM/100 µg protein) AAC-19, 1721.8±138.7; P224A, 2274.7±109.5; and A416P, 1764.4±150.8. For active transepithelial ²²Na⁺ flux assay, 60 minutes after ²²Na⁺ was added to the apical compartments, medium from basolateral compartments from each well was collected and counted. The control values are AAC-19, 3236.4±237.7; P224A, 3794.6±219.7; and A416P, 3479.4±250.8. The transepithelial electrical resistance (TER, in Ω·cm²) of monolayers was measured in culture medium and calculated by subtracting the resistance measured with the blank insert. The control TER values (in Ω·cm²) are AAC-19, 70.8±5.6; P224A, 144.6±8.9; and A416P, 96.9±7.8. Each experiment was performed in triplicate. The control values shown were from a typical experiment. n=3 to 4.

*P<0.05 and **P<0.01 vs control.
tertiary structure. In comparison to Pro222, carbonylated Pro222 might bind more strongly to c-Src SH2 domain in E1P state. Before carbonylation, both Pro222 and Ala222 were able to bind to Tyr244 of c-Src SH2 domain. After carbonylation, Pro222 was able to bind to Tyr244 as well as other amino acid residues of c-Src SH2 domain, including Asn208, Asn236, and His248. When the α1 CD2 segment (amino acid residues 152–288) and ND1 segment (amino acid residues 379–435) were used for docking analysis, it was predicted that CD2 will bind to the c-Src SH2 domain (amino acid residues 161–251) in both E1P and E2P state, and this appears to be further enhanced by Pro222 carbonylation. However, the ND1 can bind to the c-Src tyrosine kinase domain (amino acid residues 282–531) with many more posses in E1P state than in E2P state. During the E1P to E2P conformation change, the internal distance between the ND1 to pro222 in the α1 subunit was changed, as indicated by the yellow double-headed arrow (Figure 5, left and right panel), from 24.829 Å in E1P state to 6.340 Å in E2P state. Furthermore, docking analysis with Pro224 and Ala224 in Pro/Ala mutation of rat α1 showed the same predictions as seen in pig α1.

Discussion

As an ion pump, the physiological function of the Na/K-ATPase is to maintain the electrochemical sodium gradient and cellular sodium homeostasis at the expense of ATP. Recent studies have demonstrated that Na/K-ATPase also functions as a signal transducer through multiple protein–protein interactions. At physiological concentrations, binding of cardiotonic steroids such as ouabain (at low concentration without significant inhibition of Na/K-ATPase transport activity) to the Na/K-ATPase α1 subunit results in the activation of Src, transactivation of epidermal growth factor receptor (EGFR), assembly of multiple protein kinase cascades, and increases in ROS and intracellular calcium (reviewed in ). The activation of the Na/K-ATPase signaling function is largely independent of the changes in intracellular sodium concentration and significant acute inhibition of Na/K-ATPase transport activity. Functionally, this activation leads to redistribution of Na/K-ATPase and NHE3 in the RPT, resulting in a decrease in surface contents of these 2 transporters and consequently a reduction in RPT sodium reabsorption.

Our in vitro data suggest that direct carbonylation modification of the Pro222 residue in pig Na/K-ATPase α1 subunit might be a novel regulatory mechanism of Na/K-ATPase signaling. The Pro residue and α1 subunit are highly conserved (Table 1). To verify the function of Pro222 carbonylation of pig α1 subunit in our previous observation, we chose the equivalent Pro224 of rat α1 subunit to assess its role in ouabain-mediated Na/K-ATPase signaling and subsequent regulation of sodium transport. We constructed the Pro224 to Ala224 (P224A) and Ala416 to Pro416 (A416P) mutants based on a rat α1 cDNA expressing vector as we described before. Using PY-17 cells, which were derived from pig LLC-PK1 cells with an over 90% knockdown of pig α1 subunit, the ouabain-sensitive endogenous pig α1 subunit was rescued with ouabain-resistant rat α1 subunit. The remaining pig α1 subunit was further reduced by post-transfection selection with ouabain. Wild-type α1-rescued PY-17 cells (AAC-19) were used as a control. The stable P224A mutant cells (clone 9G3, abbreviated as P224A) and A416P mutant cells (clone 4, abbreviated as A416P) were selected for the following experiments. The establishment and characterization of AAC-19 and A416P cells were described before.

Figure 5. Illustration of the 3-dimensional structure of the Na/K-ATPase α1 subunit in E1P state (left panel), E2P state (right panel), and Pro222 (middle panel). From upper to lower images, Pro222, carbonylated 222, and Ala222 in Pro/Ala mutation in E1P state.
A416 is located in the nucleotide binding (N) domain of the α1 subunit. Expression of A416P mutant in PY-17 cells showed similar characteristics of AAC-19 cells, such as α1 and caveolin-1 expressions, ouabain-sensitive Na/K-ATPase activity as well as basal and ouabain-mediated c-Src activation. We reasoned that the A416P mutation can serve as a mutant control.

There are significant differences in sensitivity of the Na/K-ATPase to ouabain based on α isoforms and species. Specifically, the rodent α1 is far less sensitive than pig, dog, or human α1, largely because of the low affinity of ouabain to rodent α1. A higher concentration of ouabain (10 μmol/L) is needed to activate Na/K-ATPase-c-Src cascade and induce Na/K-ATPase endocytosis in primary culture of RPTs isolated from Dahl salt-resistant rats and AAC-19 cells. Since rat α1 is ouabain resistant, the following experiments were performed with 10 μmol/L ouabain in AAC-19 and mutant cells, which is capable of activating rat Na/K-ATPase signaling without significantly affecting the transport and enzymatic activity of the Na/K-ATPase.

Our present data demonstrated that altering carboxylation modification of Pro224 of the rat α1 subunit is able to alter Na/K-ATPase signaling and sodium handling. Rather than contributing to development and maintenance of hypertension, properly regulated RPT Na/K-ATPase signaling has a protective effect under physiological settings. The impaired RPT Na/K-ATPase-c-Src signaling cascade and induce Na/K-ATPase endocytosis in primary culture of RPTs isolated from Dahl salt-sensitive rats prompted us to investigate the role of oxidative modification in regulation of Na/K-ATPase signaling and function since an increase in oxidative stress is both a cause and consequence of hypertension and contributes to salt sensitivity.

In LLC-PK1 cells, ROS is a critical signaling mediator of ouabain-mediated RPT Na/K-ATPase/c-Src signal transduction. Specifically, carboxylation modification of Pro224 of the pig α1 subunit is involved in the regulation of Na/K-ATPase signal transduction and subsequent inhibition of transepithelial 22Na+ flux. The present data further indicated that carboxylation modification of the Pro residue is not only a key regulator but also functions as a signaling amplifier in ouabain-mediated Na/K-ATPase signals and sodium handling. Moreover, ouabain-induced ROS generation and carboxylation modification may function as the link from ouabain-Na/K-ATPase signaling to NHE3 regulation.

An increase in ROS, either induced by ouabain or glucose oxidase, stimulated Src kinase tyrosine phosphorylation and reduced protein content of Na/K-ATPase and NHE3 on the cell surface. In LLC-PK1 cells, ouabain-mediated inhibition of transepithelial 22Na+ flux was largely dependent on the coordinated regulation of basolateral Na/K-ATPase and apical NHE3 through Na/K-ATPase signaling. In LLC-PK1 cells, ouabain reduces cell surface Na/K-ATPase and NHE3 that leads to reduced apical Na+ entry through NHE3 and basolateral Na+ extrusion through Na/K-ATPase. Disruption of the Na/K-ATPase/c-Src signaling (as seen in pig α1 knockdown PY-17 cells, caveolin-1 knockout C2-9 cells, and Src kinase null SYF cells) attenuated ouabain-stimulated protein carboxylation. In control AAC-19 and mutant A416P cells, ouabain-induced inhibition of transepithelial 22Na+ flux was related to the Na/K-ATPase signaling and carboxylation. The present study is consistent with our observations in LLC-PK1 cells, suggesting that the Pro224 is a critical mediator of ouabain-stimulated Na/K-ATPase signaling. Functionally, the P224A mutation prevents ouabain-induced Na/K-ATPase signaling, protein carboxylation, and inhibition of transepithelial 22Na+ flux, further demonstrating the involvement of Pro224 and carboxylation modification in ouabain-mediated effects. The present data strengthen our hypothesis that carboxylation modification of the Pro residue is a novel regulator and an amplifier of Na/K-ATPase signaling and functions. Furthermore, these data support the hypothesis that the Na/K-ATPase/c-Src signaling complex is capable of functioning as a receptor complex of ROS by oxidative modification of the Na/K-ATPase α1 subunit like carboxylation.

It is well documented that ouabain and other stimuli stimulated c-Src activation. It has been proposed that the Na/K-ATPase α1 subunit interacts with c-Src kinase to form a functional Na/K-ATPase/c-Src signaling complex. In this model, the Na/K-ATPase α1 subunit provides the ligand binding site and the associated c-Src functions as the kinase moiety. It has also been proposed that c-Src activation is primarily due to an ATP-sparing effect based on a cell-free system. While the Na/K-ATPase inhibitors (vanadate and oligomycin) and ATP/ADP ratio regulate c-Src activation, a possible interaction between the α1 and c-Src was not addressed.

There are some additional data that are at odds with our model. It has been shown that ouabain did not induce interaction between the α1 and c-Src by immunoprecipitation assay in human breast tumor and nontumorigenic cells and ouabain-induced α1 endocytosis was independent of c-Src activation or PI(3)K in non-small cell lung carcinoma cells. We would point out that these observations are different from those that we have reported in purified pig Na/K-ATPase, primary RPT, SYF and SYF+c-Src, pig LLC-PK1, rat α1 rescued LLC-PK1, human HK-2, primary culture of human dermal fibroblasts, primary culture of rat cardiac fibroblasts, and a renal fibroblast cell line. Therefore, we do believe that the Na/K-ATPase-Src model is worthy of further exploration. Of course, it is possible that the c-Src can be activated by other

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*References 2, 5, 6, 13, 14, 18, 19, 23, 30, 32, 54, 55.
mechanisms and the Na/K-ATPase conformation change is the key step in regulating the endocytosis of this complex, per se.

Our bioinformatic analysis predicted that in pig α1 subunit, Pro222 carbonylation and Pro222/Ala mutation would not affect the enzymatic function of the Na/K-ATPase, and based on our experimental results, enzymatic function and ouabain sensitivity were unchanged by this mutation. Carbonylation of Pro222 provided more interaction possibilities with c-Src SH2 domain than native Pro222 in E1P state, favoring the interaction between the CD2 domain of α1 subunit and c-Src SH2 domain. It was also predicted that, while the α1 CD2 binds to the c-Src SH2 domain in both E1P and E2P state, which was further enhanced by Pro222 carbonylation, the α1 ND1 binds to c-Src tyrosine kinase domain with more possibilities in E1P state than in E2P state. This suggests that carbonylation might shift a balance between SH2 domain and tyrosine kinase domain binding to the former state, allowing for greater activity of the kinase. However, it is still unclear whether this effect of carbonylation of Pro222 leads to a shift from the E1P→E2P state or whether another mechanism is operant. As prevention of carbonylation at this site by our site mutation prevented activation of c-Src by ouabain, we believe that this carbonylation site may be important in the generation of signals by cardiotonic steroids. We would further speculate that the carbonylation of Pro222 might affect interactions between the α1 subunit and other signaling partners such as PI3K and the IP3R.56 However, these predictions need to be further investigated.

Prospectively, carbonylation modification of the Na/K-ATPase, induced by cardiotonic steroids and/or other stimuli, might be important in renal sodium handling. In the kidney, an increase in oxidative stress influenced a number of physiologic processes as aforementioned, including renal sodium handling.57–61 In RPTs in particular, increases in oxidative stress inhibited the activity of Na/K-ATPase and NHE3 to promote RPT sodium excretion under certain circumstances.57,59,60 Future studies will be necessary to investigate the role and mechanism of Na/K-ATPase carbonylation in renal sodium handling, especially the reversible carbonylation modification as we have previously observed.6

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**Disclosures**

None.

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Carbonylation Regulates Na/K-ATPase Signaling

Yan et al


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Protein Carbonylation of an Amino Acid Residue of the Na/K–ATPase α1 Subunit Determines Na/K–ATPase Signaling and Sodium Transport in Renal Proximal Tubular Cells

Yanling Yan, Anna P. Shapiro, Brahma R. Mopidevi, Muhammad A. Chaudhry, Kyle Maxwell, Steven T. Haller, Christopher A. Drummond, David J. Kennedy, Jiang Tian, Deepak Malhotra, Zi-jian Xie, Joseph I. Shapiro and Jiang Liu

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