Ouabain and Insulin Induce Sodium Pump Endocytosis in Renal Epithelium

Shalini Gupta, Yanling Yan, Deepak Malhotra, Jiang Liu, Zijian Xie, Sonia M. Najjar, Joseph I. Shapiro

Abstract—Cardiotonic steroids signaling through the basolateral sodium pump (Na/K-ATPase) have been shown to alter renal salt handling in intact animals. Because the relationship between renal salt handling and blood pressure is a key determinant of hypertension, and patients with insulin resistance are frequently hypertensive, we chose to examine whether there might be competition for resources necessary for receptor-mediated endocytosis. In LLC-PK1 cells, the Na/K-ATPase-α1 and carcinoembryonic antigen cell adhesion molecule 1, a plasma membrane protein that promotes receptor-mediated endocytosis, colocalized in the plasma membranes and translocated to the intracellular region in response to ouabain. Either ouabain or insulin alone caused accumulation of and carcinoembryonic antigen cell adhesion molecule 2 expression. The null renal carcinoembryonic antigen cell adhesion molecule 2 animals demonstrated greater increases in blood pressure with increases in dietary salt than control animals. These data demonstrate that cardiotonic steroids and insulin compete for cellular endocytosis resources and suggest that, under conditions where circulating insulin concentrations are high, cardiotonic steroid-mediated natriuresis could be impaired. (Hypertension. 2012;59:665-672.)

Key Words: chronic renal insufficiency ■ renal proximal tubule cell ■ endocytosis

Insulin binding induces phosphorylation of the insulin receptor (IR) carcinoembryonic antigen-related cell adhesion molecule (CEAM1) and its substrates to activate downstream signaling pathways. Phosphorylation of CEAM1, a surface membrane glycoprotein, promotes insulin endocytosis via its receptor, followed by its degradation.2 In agreement with receptor-mediated insulin uptake and downstream signaling pathways. Phosphorylation of CEAM1, a surface membrane glycoprotein, promotes insulin endocytosis via its receptor, followed by its degradation.1 In agreement with receptor-mediated insulin uptake and degradation constituting the basic mechanism of its clearance in liver and kidney, null mutation of CEAM1 impairs insulin clearance to cause hyperinsulinemia and insulin resistance.2,3

We have shown previously that cardiotonic steroids induce endocytosis of the plasmalemmal Na/K-ATPase in renal proximal tubule cells to increase urinary sodium excretion.4 Researchers have shown recently that, although some cardiotonic steroids may promote hypertension, loss of their natriuretic effects may actually exacerbate salt-dependent hypertension.5–7

Similar to IR, the epidermal growth factor receptor (EGFR) also phosphorylates CEAM1, an event that, in turn, mediates their complex formation and regulation of postreceptor signaling.8 Given that EGFR is transactivated by Na/K-ATPase-initiated signaling, the current study tests whether CEAM1 is also involving in this process.

Materials and Methods

Chemicals and Antibodies
Chemicals of the highest purity available were obtained from Sigma (St Louis, MO). Monoclonal and polyclonal antibodies against Na/K-ATPase-α1 subunit (clone C464.4), EGFR, and early endosomal antigen 1 were obtained from Upstate Biotechnology (Lake Placid, NY). Antibody against caveolin 1 (clone C060) was obtained from BD Transduction Laboratories (Lexington, KY). Monoclonal antibody against clathrin heavy chain (clone x22) was obtained from Affinity BioReagents (Golden, CO). Polyclonal antibodies against IR-β subunit, caveolin 1, c-Src, and Rab7, as well as horseradish peroxidase–conjugated goat antimouse and goat antirabbit IgG, were used for Western blots. Monoclonal antibody against the IR-β subunit was obtained from AnaSpec (Fremont, CA). Monoclonal antibody against the Na/K-ATPase-α1 subunit (clone o6F) was obtained from Developmental Studies Hybridoma Bank (University of Iowa).
of Iowa, Iowa City, IA). Normal mouse IgG and rabbit IgG were purchased from Sigma. OptiTan nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH).

Cell Culture
The pig renal proximal tubule cell line, LLC-PK1, was obtained from the American Tissue Type Culture Collection (Manassas, VA) and cultured to confluent condition as described previously. In immunostaining, LLC-PK1 cells were grown to confluence on the 24-mm polycarbonate Transwell culture filter inserts (filter pore size 0.4 μm; Costar Co, Cambridge, MA), as reported previously. LLC-PK1 cells expressing mock-vehicle (P-11, as control), Na/K-ATPase-α1 small interfering RNA (PY-17, as α1-knockdown cells), and caveolin-1 small interfering RNA (C2-7, as caveolin-1-depleted cells) were cultured in the same manner as the parent LLC-PK1 cells.

Immunofluorescence
Cells grown to confluence on the 24-mm Transwell filters were fixed and permeabilized as described by Muth et al. The cells were then probed with primary antibody for 90 minutes at room temperature or overnight at 4°C (monoclonal anti-α1 antibody, Upstate; polyclonal anti-CEACAM1 antibody, 1:100 dilution in goat serum dilution buffer). After 3 washes with permeabilization buffer, the cells were incubated with Alexa Fluor 546-conjugated or Alexa Fluor 488-conjugated secondary antibody for 1 hour at room temperature. After 3 additional washes, specimens were mounted using Prolong Antifade medium (Molecular Probes, Eugene, OR). All of the images were acquired via a Leica TCP SP5 broadband confocal microscope system (Leica, Mannheim, Germany) with a ×63 oil-immersion objective and analyzed with Leica software. The confocal microscope studies were performed using resources of the Advanced Microscopy and Imaging Center at the University of Toledo Health Science Campus.

Immunoprecipitation
Immunoprecipitation experiments were performed as described by Chibaln et al and published previously from our laboratory. LLC-PK1 cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer. Insoluble material was removed by centrifugation (14,000×g 10 minutes at 4°C). Aliquots of supernatant (total of 1 mg of protein) were immunoprecipitated overnight at 4°C with monoclonal anti-Na/K ATPase-α1 subunit antibody (Upstate). Immunoprecipitates were washed with protein G-agarose beads, rotating for 2 hours at 4°C. Beads were washed 5 times in radiolmmunoprecipitation assay lysis buffer, and immunoprecipitated proteins were eluted with Laemmli sample buffer and analyzed by Western blot.

Preparations of Endosomes
Endosomes were fractionated on a floating gradient using the technique of Gorvel et al. The early endosomal fraction was collected at the 16% to 10% sucrose interface. The identity of early endosomal fractions was determined with antibodies against early endosome protein marker early endosomal antigen 1, as we have described previously.

Western Blot
Immunoblotting was performed as described previously. Briefly, cell lysates (50 μg per lane), endosomal fractions (15 μg per lane), or immunoprecipitates (from 1 mg total protein per sample) were separated by 4% to 15% gradient SDS-PAGE (BioRad) and transferred to nitrocellulose membrane. After transfer, membranes were blocked with 5% milk in Tris-buffered saline-Tween (Tris-HCl 10 mmol/L, NaCl 150 mmol/L, Tween 20, 0.05%; pH 8.0) for 1 hour at room temperature, and immunoblotting was performed. Detection was performed with the enhanced chemiluminescence Plus Western Blotting Detection System (Amersham, Buckinghamshire, United Kingdom). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. Autoradiograms were scanned with a Bio-Rad GS-670 imaging densitometer (Bio-Rad, Hercules, CA) to quantify signals.

Animals
The generation of Ceacam2-null (Cc2−/−) mice was described previously. Male mice from 4 to 8 months of age were studied. Animals were kept in a 12-hour dark/light cycle and fed standard regular chow and tap water ad libitum. All of the procedures were approved by the Institutional Animal Care and Utilization Committee.

Statistical Analysis
Data are presented as the mean±SEM. Data obtained were first tested for normality and then subjected to parametric analysis. For comparison of more than 2 groups, 1-way ANOVA was used using the Student t test with Bonferroni correction for multiple comparisons for post hoc analysis. Statistical analysis was performed with SPSS software.

Results
Na/K-ATPase-α1 and CEACAM1 Colocalize to the Plasmalemma of LLC-PK1 Cells
To determine whether CEACAM1 protein is expressed in porcine renal proximal tubule LLC-PK1 cells, an in vitro
model extensively used in our laboratory, we initially carried out Western analysis of whole-cell lysates. CEACAM1 protein was easily detected in LLC-PK1 cells, albeit to a much lower extent than rat hepatoma H4IIE cells (data not shown). Subsequently, immunofluorescence analysis demonstrated that CEACAM1 was mostly localized to the plasmalemma of LLC-PK1 monolayers grown on Transwell membranes (Figure 1, top row, green fluorescence).

This expression pattern of CEACAM1 was comparable to that of the Na/K-ATPase-β subunit (Figure 1, top row, red fluorescence).

Figure 2. A. Autoradiographs of representative Western blot for carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1, epidermal growth factor receptor (EGFR), and insulin receptor (IR)-β expression in early endosomal fractions of renal proximal tubule LLC-PK1 cells treated with ouabain (100 nmol/L, 30 minutes). B through D, Corresponding quantitative data as the mean±SEM of 5 experiments. Early endosomal antigen 1 (EEA1), an early endosomal marker, was used as loading control. *P<0.05 vs control. C indicates control; O, ouabain.

Figure 3. Representative Western blots and quantitative protein expression shown as the mean±SEM of 5 samples for carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1 (A and B), epidermal growth factor receptor (EGFR; C and D), and insulin receptor (IR)-β (E and F) expression in early endosomal fractions of renal proximal tubule P11, PY-17, and C2–7 cells treated with exogenous ouabain (100 nmol/L) and/or insulin (100 nmol/L) for 30 minutes. Early endosomal antigen 1 (EEA1) was used as loading control. *P<0.05 vs control. C indicates control; I, insulin; O, ouabain; O+I, ouabain+insulin. □, CEACAM1; □, IR-β; □, EGFR.
red fluorescence), as we and others have reported previously.17 Furthermore, both of these proteins colocalized, as revealed by merging analysis (Figure 1, top row, yellow fluorescence).

Ouabain- and Insulin-Induced Accumulation of CEACAM1 in Early Endosomal Fractions of LLC-PK1 Cells

Ouabain (100 nmol/L, 30 minutes) increased intracellular content of the Na/K-ATPase-α1 subunit in LLC-PK1 cells (Figure 1, bottom row, red fluorescence). Intracellular CEACAM1 content also increased in response to the same ouabain treatment (Figure 1, bottom versus top row, green fluorescence). These data suggested that the α1 subunit and CEACAM1 are coexpressed on the plasmalemma of LLC-PK1 cells, and both undergo internalization in response to ouabain.

Western blot analysis revealed the presence of CEACAM1 in the early endosomal fractions of LLC-PK1 cells (Figure 2A). In comparison with control cells, ouabain treatment (100 nmol/L, 30 minutes) causes increases in early endosomal content of CEACAM1 by ≈2.5-fold (Figure 2A and 2B), IR-β subunit by ≈3-fold (Figure 2A and 2C), and EGFR by ≈1.5-fold (Figure 2A and 2D). Ouabain also increased the early endosomal content of the α1 subunit of the Na/K-ATPase, as we have reported previously12 (data not shown).

We next investigated whether insulin exerts a synergistic effect with ouabain on CEACAM1 internalization and whether CEACAM1 internalization depends on Na/K-ATPase signaling. To address this question, we used LLC-PK1 cells stably transfected with mock-expressing vector alone (P11) or with small interfering RNA expressing vectors of knockdown Na/K-ATPase α1-subunit expression (PY-17) or knockout caveolin 1 expression (C2-7).18 Like ouabain,
insulin (100 nmol/L, 30 minutes) induced early endosomal accumulation of CEACAM1, IR-β, and EGFR proteins in P11 control cells (Figure 3A and 3B). However, concurrent treatment with ouabain and insulin did not produce synergistic endocytic effects in P11 cells (Figure 3A and 3B).

In the PY-17 and C2–7 cells where we have previously shown ouabain signaling through the Na/K-ATPase to be minimal, insulin, but not ouabain, induced the endosomal accumulation of these receptors and CEACAM1 (Figure 3C through 3F). Moreover, ouabain failed to influence the effect of insulin on the internalization of these proteins in both PY-17 and C2–7 cells.

**Na/K-ATPase-α1 and IR-β Colocalize to the Plasmalemma of LLC-PK1 Cells**

Immunofluorescence analysis demonstrated that sodium pump-α1 and IR-β subunits were localized to the plasmalemma of monolayer LLC-PK1 cells grown on Transwell membranes.

Ouabain increased internalization of the α1 subunit as before, and insulin treatment, with or without ouabain, also caused internalization of both α1 and IR-β subunits (Figure 4).

**Ouabain- and Insulin-Induced Accumulation of Sodium Pump-α1 Subunits in Early Endosomal Fractions of LLC-PK1 Cells**

Consistent with our previous reports, ouabain increased sodium pump-α1 subunit protein accumulation in early endosomal fraction of LLC-PK1 cells. Interestingly, insulin alone also caused early endosomal α1 subunit accumulation, but concurrent ouabain and insulin treatment was neither synergistic or additive (Figure 5A and 5B).

**Ouabain- and Insulin-Induced c-Src Phosphorylation in LLC-PK1 Cells**

As above, LLC-PK1 cells were treated with ouabain and/or insulin for 30 minutes, and c-Src phosphorylation was determined in LLC-PK1 whole-cell lysates and early endosomal fractions. Quantitative data are shown as the mean ± SEM of 5 experiments. *P < 0.05 vs control; **P < 0.01 vs control. C indicates control; I, insulin; O, ouabain; O+I, ouabain + insulin.
mined. Relative to control, ouabain stimulated c-Src phosphorylation in whole-cell lysates by ~2.5-fold (Figure 6A and 6B), as well as in early endosomal fractionations (Figure 6C and 6D). Interestingly, insulin induced a comparable effect to that of ouabain on c-Src phosphorylation in early endosomal fractions (Figure 6C and 6D). Concomitant treatment with insulin and ouabain produced no synergistic induction on c-Src phosphorylation in either whole-cell lysates (data not shown) or early endosomal fraction (Figure 6C and 6D).

Effect of Ouabain and Insulin on Interaction Among Na/K-ATPase-α1, CEACAM1, and Caveolin-1 in LLC-PK1 Cells

To elucidate whether there is an interaction between α1 subunit and CEACAM1, LLC-PK1 cells were treated with ouabain and/or insulin for 1 hour and immunoprecipitated with anti-Na/K-ATPase-α1 subunit antibody. Immunoblotting with antibodies against CEACAM1 and α1 (as control) revealed that ouabain treatment enhanced the interaction between CEACAM1 and the α1 subunit (Figure 7A and 7B). Insulin similarly augmented α1-CEACAM1 coprecipitation, although this did not reach statistical significance (Figure 7A and 7B). Concurrent treatment with insulin and ouabain induced a mild statistically significant increase in α1-CEACAM1 coprecipitation (Figure 7A and 7B). Immunoblotting with an antibody against caveolin 1 revealed that ouabain and insulin increased the coimmunoprecipitation of the Na/K-ATPase-α1 subunit and caveolin 1 and that this effect of insulin was not synergistic with ouabain (Figure 7A and 7C).

Salt Increased Systolic Blood Pressure in Mice With Null Mutation of Cceam2 (Cc2−/−)

CEACAM2, a highly homologous related protein to CEACAM1, is the predominant protein in the murine kid-

<table>
<thead>
<tr>
<th>Variable</th>
<th>0.4% Salt Diet, Day 8</th>
<th>2.0% Salt Diet, Day 8</th>
<th>4.0% Salt Diet, Day 8</th>
<th>8.0% Salt Diet, Day 8</th>
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<td>Cc2−/−</td>
<td>WT</td>
<td>Cc2−/−</td>
<td>WT</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.24±0.76</td>
<td>28.51±0.91</td>
<td>28.84±1.04</td>
<td>28.95±1.05</td>
</tr>
<tr>
<td>Urine output, mL/24 h</td>
<td>0.9±0.1</td>
<td>0.6±0.2</td>
<td>2.6±2.0</td>
<td>3.1±0.8</td>
</tr>
<tr>
<td>UNaV, μeq/24 h</td>
<td>53±5</td>
<td>49±7</td>
<td>405±82</td>
<td>477±92</td>
</tr>
</tbody>
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Data are shown as the mean±SEM of measurements performed on 5 animals in each group. WT indicates wild-type; Cc2−/−, Cceam2-null; UNaV, urinary sodium excretion.

Discussion

It is well established that ouabain binds to the Na/K-ATPase and induces its endocytosis via a signaling cascade, whereas binding of insulin to its receptor induces association of the receptor with CEACAM1 and internalization for ligand degradation and IR recycling.1,21,22 CEACAM1 is also present on the plasmalemma in various types of cells, where it can

Figure 8. Effect of high-salt diet (2%, 4%, or 8%) vs control (0.4%) on systolic blood pressure (mm Hg) in mice lacking carcinoembryonic antigen-related cell adhesion molecule 2 (Cc2−/−); ■ vs wild-type (WT); □. Data are shown as the mean±SEM of measurements performed on 5 animals in each group. *P<0.05 vs WT.
interact with the IR via Shc adaptor protein.\cite{24-26} Our data suggest that both the sodium pump and CEACAM1 are capable of responding to cardiotonic steroids in the renal proximal tubule, a crucial site of blood pressure regulation and insulin clearance. It has also been shown that the IR and CEACAM1 are expressed on both apical and basolateral aspects of polarized Madine-Darby canine kidney cells, although predominantly on the basolateral surface.\cite{24-26} Ouabain treatment of LLC-PK1 cells and a high-salt diet result in the coordinated redistribution of the apical sodium proton antiporter NHE3 through mechanisms that are yet to be elucidated.\cite{27,28} As well as decreases in NHE3 expression on a transcriptional level.\cite{29} It is the redistribution of the NHE3 that is felt to be rate limiting in terms of proximal tubular sodium handling or natriuresis, and our previous data suggest that this is a consequence of Na/K-ATPase signaling through Src.\cite{27,29} Although insulin has been shown to initiate phosphorylation of the sodium pump in skeletal muscle,\cite{30} our current findings suggest that the sodium pump and IR compete for resources necessary for endocytosis. What is not clear from our data is whether insulin induces identical changes in proximal tubular sodium handling or whether redistribution of the NHE3 and other transport proteins are affected differently. Further studies will be necessary to explore this important area.

We also observed that the renal CEACAM molecule in mice (CEACAM2) is involved in cardiotonic steroid-induced Na/K-ATPase endocytosis and, hence, contributes to blood pressure regulation. Interestingly, we have reported recently that, in the Dahl salt-sensitive strain of rat, impaired proximal tubular Na/K-ATPase endocytosis in response to salt loading in vivo or ouabain exposure in vitro can be demonstrated in comparison with the Dahl salt-resistant strain. In other words, the lack of renal naturopic response to circulating cardiotonic steroids corresponded with the increases in blood pressure seen in the Dahl salt-sensitive strain with salt loading.\cite{31} These data are consistent with the observed hypertension seen in the Cc2\(^{-/-}\) mice subjected to a high-salt diet. However, as mentioned above, additional studies are clearly required to further characterize abnormalities in insulin or cardiotonic steroid-induced signaling through the Na/K-ATPase “signalosome”\cite{32-34} in the development of salt-sensitive hypertension in these Cc2\(^{-/-}\) mice.

**Perspectives**

The current studies demonstrate that ouabain-induced signaling through the sodium pump also interacts with IR-associated molecules, including CEACAM, in the kidney. Although further studies are required, the data identify a competition between ouabain- and insulin-mediated endocytosis and suggest a potential role for this competition in the regulation of salt excretion and, therefore, the pathogenesis and possibly treatment of hypertension seen in hyperinsulinemic states.

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**Disclosures**

None.

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