Sodium loading changes urinary protein excretion: a proteomic analysis

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Submitted 15 April 2002; accepted in final form 6 February 2003

R E N A L  S O D I U M  H A N D L I N G  I S  I M P O R T A N T  i n  m a i n t a i n i n g  i n t r a c e l l u l a r  a n d  e x t r a c e l l u l a r  s o d i u m  a n d  w a t e r  h o m o e o s t a s i s .  R e n a l  s o d i u m  e x c r e t i o n  i s  c o n t r o l l e d  b y  c h a n g e s  i n  b o d y  f l u i d  v o l u m e  a n d  t h e  s t i m u l a t i o n  o f  o s m o r e c e p t o r s  (1, 5) .  T h e  m e c h a n i s m s  t h a t  o c c u r  i n  t h e  k i d n e y  c a n  b e  d i v i d e d  i n t o  t h r e e  m a i n  c a t e g o r i e s ; h u m o r a l  s i g n a l s  (e s p e c i a l l y  v a s o p r e s s i n  a n d  t h e  r e n i n - a n g i o t e n s i n  s y s t e m) ,  r e n a l  n e r v e  a c t i v i t y ,  a n d  p h y s i c a l  f a c t o r s  (15) .  P a s s i v e  a n d  a c t i v e  s o d i u m  t r a n s p o r t e r s  a l o n g  r e n a l  t u b u l e s ,  m o s t l y  a t  p r o x i m a l  t u b u l e s ,  a r e  g e n e r a l l y  c o n s i d e r e d  a s  t h e  t a r g e t s  o f  t h e s e  m e c h a n i s m s .  H o w e v e r ,  a  c o h e r e n t  u n d e r s t a n d i n g  o f  t h e s e  c o m p l e x  p a t h w a y s  a n d  t h e  r o l e s  o f  r e g u l a t o r y  t a r g e t s  o t h e r  t h a n  t h e s e  t r a n s p o r t e r s  i s  l a c k i n g .

A sodium-excess status induced by salt loading causes changes in the renal expression of several proteins, which can be divided into two main groups; sodium excretion-controlling proteins (8, 9) and regulated proteins that cause systemic effects (17, 19, 20, 29, 30). Previous studies have demonstrated that aquaporin-2, Na⁺/H⁺ exchanger type 3 (NHE3), Na⁺-/K⁺-/2Cl⁻ cotransporter, and thiazide-sensitive Na⁺-/Cl⁻ cotransporter were excreted into the urine (7, 14, 21). Western blotting and RIA were used to identify expression of the proteins in those studies. However, these techniques are limited by the relatively small number of proteins that can be studied for each experiment and the need for specific antibodies to those proteins. In addition, antibody-based studies only identify proteins that are suspected to be there a priori. Other tubular proteins are also excreted into the urine, and sodium loading may alter urinary excretion of unsuspected proteins that are involved in sodium homeostasis. The global study of a large complement of urinary proteins may contribute to understanding renal sodium handling.

In 1975, O’Farrell (18) developed a technique for the resolution of proteins using two-dimensional PAGE (2D PAGE or 2-DE), and 1,100 proteins from Escherichia coli were visualized. Using this technique, a large number of proteins can be studied simultaneously without specific antibodies. The proteins are separated by isoelectric point (pI) in the horizontal dimension and by molecular weight (MW) in the vertical dimension. The protein spots can be visualized by several staining methods. Recently, up to 10,000 protein spots have been visualized by high-resolution 2-DE (12). The analysis of separated proteins by mass...
spectrometry has permitted analysis of proteins on a “genomic” scale (11). The analysis of proteins on a genomic scale has acquired the name “proteomics” (2). A common approach for proteomic analysis uses resolution of proteins by high-resolution 2-DE, peptide mass fingerprinting, and bioinformatics to identify the proteins in a high-throughput fashion. Once the proteins are visualized, the protein spots are excised and undergo in-gel tryptic digestion. Peptide masses are obtained by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS). Peptide mass fingerprinting is then performed to identify the protein, using search engines to match the peptide masses to the theoretical masses in protein databases. The National Center for Biotechnology Information (NCBI) is an annotated protein database containing more than 1.2 × 10^6 peptide sequences and 3.9 × 10^8 residues (http://www.ncbi.nlm.nih.gov).

We used proteomic analysis to determine alterations in urinary protein excretion during acute sodium loading. A self-controlled study was conducted in young male Sprague-Dawley rats fed with deionized (di) water that was then replaced with 2.7% NaCl. Urinary sodium excretion was significantly increased, and urinary excretion of several proteins was altered after acute sodium loading.

**MATERIALS AND METHODS**

**Urine collection.** All studies using rats were approved by the University of Louisville Institutional Animal Care and Use Committee. A self-controlled study was conducted in four young male Sprague-Dawley rats (body wt 383 ± 16 g). The rats were transferred to metabolic cages and fed with 18% (18 MJ) water and rat chow obtained from PMI Nutrition (Richmond, IN) for 24 h. Twenty-four-hour urine was collected for urinary Na^+^ concentration measurement. The rats were then transferred to another cleaned metabolic cage with di water without any food (to prevent contamination with proteins from food particles). Four-hour urine for protein analysis was collected with a protease inhibitor cocktail (0.1 mg/ml leupeptin, 0.1 mg/ml PMSF, and 1 mM sodium azide in 1 M Tris, pH 6.8). The rats were then immediately changed to 2.7% NaCl feeding, and the rat chow was returned. Urine was collected over 24 h for urinary Na^+^ concentration measurement. After 24 h, the rats were then transferred to cleaned metabolic cages for 2.7% NaCl feeding but no food. Four-hour urine for protein analysis was again collected with a protease inhibitor cocktail. Urine protein collections were done at the same time of day to avoid diurnal variation.

**Urineary Na^+^ concentration measurement.** Urinary Na^+^ concentration measurement was performed with an indirect ion-specific electrode on a Beckman Coulter LX20.

**Sample preparation.** The samples were passed through a 0.34-mm Whatman chromatography paper and then centrifuged at 1,000 g for 5 min. The supernatants were saved and centrifuged at 200,000 g for 120 min. The pellets were resuspended in 100 μl of 250 mM sucrose in 10 mM triethanolamine. The concentration of proteins was measured by spectrophotometry using a protein microassay (Bio-Rad Laboratories, Hercules, CA) based on Bradford’s method (6).

**First dimension of 2-DE.** A tube gel mobile ampholyte running system (Genomic Solutions, Ann Arbor, MI) was used for first-dimensional isoelectric focusing. The cathode buffer was 100 mM sodium hydroxide, and 10 mM phosphoric acid was used as an anode buffer. Precast carrier ampholyte tube gels [pH 3–10, 1 mm × 18 cm (Genomic Solutions)] were prefocused with a maximum of 1,500 V and 110 μA/tube. A total of 50 μg from each sample was loaded into each tube and was focused for 17 h, 30 min to reach 18,000 volt hours.

**Second dimension of 2-DE.** The gels were extruded from the tubes after completion of focusing and were incubated in premixed Tris/acetate equilibration buffer with 0.01% bromophenol blue and 50 mM DTT for 2 min. The tube gels were then loaded onto precast 8–18% gradient, 22 × 22-cm slab gels (Genomic Solutions). Lower running buffer contained 25 mM Tris base, 192 mM glycine, and 0.1% SDS. Upper running buffer was a 2× solution of the lower buffer. The system was run with a maximum of 500 V and 20,000 mW/gel.

**Sypbro ruby staining and visualization.** The gel slabs were fixed in 10% methanol and 7% acetic acid for 30 min. The fixative solution was removed, and 500 ml of Sypbro gel stain (Bio-Rad Laboratories) were added to each gel and incubated on a continuous rocker at room temperature for 18 h. A high-resolution, 12-bit camera with a UV light-box system (Genomic Solutions) was used to visualize the protein spots with an optimal exposure time point of 3 s. The images were digitally inverted before analysis with 2D software.

**Matching and analysis of protein spots.** Investigator HT analyzer (Genomic Solutions) software was used for matching and analysis of spot expression on the gels. A representative gel was constructed as a reference for each group. An average mode of background subtraction was used for normalization of intensity volume on each gel and for compatibility of the intensity among gels. The data were reported as “normalized intensity,” which were corrected by total intensity of all spots from all the gels, instead of the raw intensity values being used. This normalized value provides a ratio-metric comparison of protein abundance. The representative gel was then used for determination of existence and difference of protein expression between groups.

**In-gel tryptic digestion, MALDI-TOF MS, and peptide mass fingerprinting.** In-gel tryptic digestion and sample preparation for MALDI-TOF MS were performed as described previously by our laboratory (3, 25). Peptide mass fingerprinting was used for protein identification from tryptic fragment sizes by using the Mascot search engine (http://www.matrixscience.com). The search was based on the entire NCBI protein database on the assumption that peptides are monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. Mass tolerance of 150 parts/million (ppm) was the window of error allowed for matching the peptide mass values. Probability-based MW search scores were estimated by a comparison of search results against an estimated random match population and were reported as −10 log10(P), where P is the absolute probability. Scores >71 were considered significant (P < 0.05). Protein identities with scores less than the significant level were reported as unidentified.

**Prediction of posttranslational modifications.** Potential posttranslational modifications (PTMs) were predicted using the FindMod search engine (http://ca.expasy.org/tools/ findmod/). Because the presence of a PTM causes a peptide mass shift, the potential PTMs can be predicted by matching the mass difference (mass difference = theoretical mass – observed mass) to the masses of known PTMs. To date, there are at least 30 known PTMs provided in the database. A window of error (Δmass) of 150 ppm was allowed.
Western blotting. Urinary proteins were processed as for 2-DE analysis. SDS sample buffer (Tris-HCl, glycerol, SDS, DTT, and bromophenol blue) was added 1:1 to the protein solution. The mixture was heated at 100°C for 5 min. The protein concentration of each sample was measured by the spectrophotometric method using the HP 8453 UV-visible system (Hewlett-Packard, Palo Alto, CA) and Bio-Rad Protein Assay (Bio-Rad Laboratories), and 20 μg of total proteins were equally loaded onto each lane on 10% SDS-PAGE gels. Proteins on the gel were transferred to a nitrocellulose membrane by electroblotting. The membrane was incubated with mouse monoclonal anti-ezrin (Sigma, St. Louis, MO) 1:1,000 in 5% milk/Tween 20 Tris-base sodium (TTBS) at 4°C overnight. Immunoreactive proteins were detected by radiography using IgG conjugated with horseradish peroxidase. The membrane was then stripped in 0.2 N NaOH for 5 min and rebotted with goat anti-mouse albumin (Bethyl Laboratories, Montgomery, TX) 1:1,000 in 5% milk/TTBS, mouse monoclonal anti-actin (A4700, Sigma) 1:200 in 5% milk/TTBS, and mouse monoclonal anti-calbindin-D28K (Sigma), respectively. The intensity analyses of immunoreactive bands were performed using a PDSI Densitometer (Amerham Biosciences, Piscataway, NJ).

Statistical analyses. The Mann-Whitney test (version 10.0, SPSS) was used for a comparison of the differences between two groups. Exact and Monte Carlo resampling methods were used to reassign the data for multiple analyses of a single data set. Therefore, Exact and Monte Carlo P values were calculated on the basis of the permuted data, adjusted for multiple inferences, and corrected for tied values. Only Exact P values < 0.05 with an agreement with a Monte Carlo test were considered statistically significant. This significance level is based on the reassignment of a test statistic, which is more accurate than using asymptotic significance values when the sample size is small (4). To avoid changes by chance or normal variability, only changes greater than two-fold (0.5-fold less or 2-fold greater than the control) were considered significant. The data are reported as means ± SE.

RESULTS

The animals were fed with dl water for 28 h and then with 2.7% NaCl for 28 h. Urinary Na⁺ concentration was measured over the first 24 h of each phase. All of the animals were in a sodium-excess status as determined by an increase in urinary Na⁺ concentration and 24-h urinary Na⁺ (Table 1). After the first 24 h of each phase, the animals were transferred to another clean metabolic cage without rat chow to avoid contamination of dietary proteins from food particles. Preliminary data showed that contamination of proteins from food particles could interfere with analysis of urinary proteins because proteins in food particles incubated in water were seen on gels (data not shown). We collected the urinary samples in clean metabolic cages without the presence of food to eliminate this problem. Urinary proteins were resolved by 2-DE as outlined in MATERIALS AND METHODS.

Up to 277 protein spots were visualized by Sypro ruby staining on each gel (Fig. 1). HT analyzer 2D software was employed to measure and compare spot intensity, which represents the amount of protein per spot. Differentially expressed protein spots were excised and underwent in-gel tryptic digestion. Figure 2A demonstrates mass spectra of peptide masses obtained by MALDI-TOF MS from spot 1 in Fig. 1. Peptide mass fingerprinting was then performed to identify the proteins by using the Mascot search engine, as demonstrated in Fig. 2B. The peptide masses shown in Fig. 2A, A and B, were significantly matched with the theoretical masses of the protein NEP 24.11 (P < 0.05).

Urinary excretion of 45 protein components was significantly changed after acute sodium loading, as summarized in Table 2. Only significant changes, 0.5-fold less or 2-fold greater than control, were included. Forty-one protein components were significantly decreased, whereas only four components were increased in intensity after acute sodium loading. Peptide mass fingerprinting did not identify 5 of the 45 differentially expressed spots. All of the identified spots had probability-based protein MW search scores > 71 (P < 0.05). Most of the matching results contained no missed cleavage sites by trypsin, and a window of error was much less than 150 ppm. The expected pl and MW of the identified proteins corresponded with their positions in the 2D gels. GenInfo identification numbers in the NCBI database are also provided in Table 2. Of the identified spots, several spots were in a series of the same protein with similar MWs but different plS, suggesting PTMs. We predicted several PTMs in these proteins by bioinformatic analyses using the FindMod search tool (Table 3).

Although we have shown in previous studies that the data obtained from proteomic analysis are consistent with other standard conventional methods (3, 24), we also confirmed the proteomic data by Western blot analyses in the present study. Western blotting for ezrin clearly showed that excretion of ezrin was significantly decreased after acute sodium loading, consistent with the proteomic data (Fig. 3A). Conversely, the level of albumin was increased by acute sodium loading (Fig. 3B). We hypothesized that using strict analytic criteria in the present study likely caused us to underestimate the number of proteins that were changed. To address this hypothesis, we examined the effect of acute sodium loading on excretion of two other abundant proteins in the kidneys, actin and calbindin (3). Urinary excretion of actin was decreased, but excretion of calbindin was increased after acute sodium loading (Fig. 3, C and D).

Table 1. Urinary parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>dl</th>
<th>2.7% NaCl</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>U Na⁺, mmol/l</td>
<td>131.5 ± 21.6</td>
<td>435.5 ± 32.4</td>
<td>0.014</td>
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<tr>
<td>24-h U volume, ml</td>
<td>13.78 ± 2.90</td>
<td>10.00 ± 3.39</td>
<td>0.371</td>
</tr>
<tr>
<td>24-h U Na⁺, mmol/24 h</td>
<td>1.70 ± 0.27</td>
<td>4.53 ± 1.74</td>
<td>0.029</td>
</tr>
<tr>
<td>24-h U protein, mg</td>
<td>1.06 ± 0.10</td>
<td>0.95 ± 0.02</td>
<td>0.171</td>
</tr>
</tbody>
</table>

Values are means ± SE. dl, deionized; U Na⁺, U volume, U Na⁺, and U protein; urinary Na⁺ concentration, volume, Na⁺ content, and protein, respectively. U protein was calculated from 4-h urinary collection in the absence of food (preliminary data showed that contaminated food particles in the urine affected the protein level).
DISCUSSION

We used proteomic analysis to study changes in the relative abundance of a large number of urinary proteins simultaneously. Using 2D analysis software, protein expression could be compared based on the intensity of staining, which represented the amount of protein per spot. Acute sodium loading caused changes in urinary excretion of 45 protein components. Some of the altered proteins have never been studied in relationship to salt loading, and their roles in sodium regulation have not been established. Several proteins, such as NEP 24.11 (enkephalinase or neprilysin), solute carrier family 3, meprin 1α, and ezrin (villin-2), are typical membrane proteins. Some proteins, such as vacuolar H⁺-ATPase, dnaK-type chaperone (heat shock protein 72), albumin precursor, and chloride intracellular channel protein 1, were identified in rat kidney cortex and medulla in our previous study (3). Cells, debris, and particles were completely removed from the samples, as de-
termined by a hemacytometric counting chamber. Thus most of these proteins probably originate in the renal tubules and may play important roles in sodium regulation.

High-throughput proteomic technologies allow a large number of proteins to be studied simultaneously. Proteomic analysis may lead to the identification of both expected changes and unexpected changes in an experimental condition. One of the strengths of proteomic analysis is that identification of coordinated changes in protein expression may lead to more focused hypotheses in physiology and pathophysiology. This is illustrated by our previous work, in which we used proteomic analysis to generate a new hypothesis to explain hypertension induced by intermittent hypoxia (EH) in an animal model (24). This hypothesis was then strongly supported by demonstrating that transgenic hKLK1 rats, which overexpress human renal kallikrein, are resistant to EH-induced hypertension. Transgenic hKLK1 animals were protected from EH-induced hypertension (24). A similar approach can be applied to proteomic data such as those produced in the present study.

In the present study, we performed expression proteomics to demonstrate changes in protein excretion after acute sodium loading. Several hypotheses and new insights regarding renal sodium handling can be generated from these proteomic data. An example is a potential role of ezrin in renal sodium regulation. Ezrin (villin-2) is a member of the ezrin/radixin/moesin family of actin-binding proteins, which function as membrane-cytoskeletal cross-linkers (13). Ezrin colocalizes and closely associates with NHE3 and Na+/H+ exchanger regulatory factor (NHERF) (27). Ezrin binds...
with actin and NHERF and forms a multiprotein complex with NHE3. Formation of this complex facilitates NHE3 phosphorylation and inhibits Na+/H+ exchange, resulting in inhibition of NaCl and NaHCO3 reabsorption in the proximal tubules (22). Change of ezrin expression obtained from proteomic analysis in the present study was not spurious or by chance. Western blotting for ezrin clearly confirmed a decrease in urinary ezrin excretion after acute sodium loading. Functional proteomics and other physiological studies are needed to determine roles of the altered proteins in renal sodium regulation.

Actin is a cytoskeletal protein that plays an important role in cell signaling and cytoskeletal assembly. The role of actin in epithelial and renal tubular sodium transport has previously been established (10, 16, 23). Alterations in renal expression of actin by hypertension (24) and by other experimental conditions (Thongboonkerd V and Klein JB, unpublished observations) were also shown in our previous studies. Additionally, actin binds to ezrin as a part of membrane-cytoskeletal cross-linkers (13). Therefore, a change in actin excretion in the present study was not unexpected. To test that the decreased excretion of ezrin and actin (Fig. 3, 10).
A and C) was not the result of a smaller amount of protein loaded in the NaCl lanes of PAGE, we performed Western blot analyses for albumin and calbindin, the two proteins that are not involved in ezrin/radixin/moesin-actin assembly. Excretion of albumin and calbindin was increased after acute sodium loading (Fig. 3, B and D). Indeed, the equal amount of protein loaded in each PAGE lane was controlled by spectrophotometry before immunoblotting procedures were begun.

Protein modification is one of the regulators of protein function. Proteomic approaches provide information about PTMs that is not obtained by many other methods. We used the FindMod tool to predict potential PTMs of the identified proteins. PTMs cause changes in protein pI, leading to the presentation of a row of multiple protein spots of the same protein. This phenomenon is observed not only with regard to urine (26) but also serum and other body fluids (28).

We also have some concerns regarding an interpretation of the results in the present study. First, several possible physiological explanations exist for differential urinary excretion of proteins. A protein that is necessary could have increased expression, leading to

### Table 3. Potential PTMs

<table>
<thead>
<tr>
<th>Potential PTMs</th>
<th>NEP 24.11</th>
<th>Solute Carrier</th>
<th>Meprin 1α</th>
<th>Diphor-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Deamidation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycation</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methylation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dimethylation</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Trimethylation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Palmitoylation</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pyrroloidal</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methylation</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Potential PTMs were predicted by bioinformatic analysis using the FindMod search tool. Because PTMs generally cause a peptide mass shift, the potential PTMs can be predicted by matching the mass difference (mass difference = theoretical mass – observed mass) to the masses of known PTMs.

![Fig. 3](image-url)

**Fig. 3.** Western blot analyses. Western blot analyses show decreased excretion of ezrin (A) and actin (C) and increased excretion of albumin (B) and calbindin (D). A total of 20 μg of protein was equally loaded in each lane (n = 3 animals).
increased appearance in the urine. Alternatively, a necessary protein could be retained in the cell, leading to decreased appearance in the urine. Second, we could not identify some known sodium transporters in the present study. Protein identification using 2-DE and MALDI-TOF is limited by the sensitivity of these techniques. Either using greater amounts of proteins loaded onto 2-DE, utilizing additional prefractionated steps, or applying a more sensitive technique, such as liquid chromatography followed by tandem MS (LC-MS/MS), may be the solution. Another concern is the strict criteria we used to determine significant changes to avoid false-positive results. The quantities of most of the proteins were decreased by sodium loading, whereas those of only a few proteins were increased. The quantities of several proteins tended to increase, but the increase was not statistically significant. Use of these strict criteria likely caused us to underestimate the number of proteins that were changed because we tested for actin and calbindin by immunoblotting (Fig. 3, C and D). Our findings in the present study represent only the “tip of the iceberg” for the entire number of changes in urinary protein excretion caused by acute sodium loading.

In summary, we used proteomic analysis to determine global alterations in urinary protein excretion during acute sodium loading. Several proteins that play important roles in the transport of sodium and other solutes, cellular pH regulation, and other cellular functions were involved in this response. Several hypotheses can be generated from these data. Further functional studies are needed to determine the coordination of these regulated proteins and their complex mechanisms in renal sodium handling.

This work was supported by the Carl W. Gottschalk Research Scholar Award from the American Society of Nephrology (to J. M. Arthur), National Institutes of Health Grants 21-DK-629686–01 and R01-HL-66358–01, and the Department of Veterans Affairs, Louisville, KY (to J. B. Klein). V. Thongboonkerd is a recipient of an International Fellowship Training Award from the International Society of Nephrology and from the National Kidney Foundation of Thailand.

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