Proteomic profiling of the effect of metabolic acidosis on the apical membrane of the proximal convoluted tubule

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Walmsley SJ, Freund DM, Curthoys NP. Proteomic profiling of the effect of metabolic acidosis on the apical membrane of the proximal convoluted tubule. Am J Physiol Renal Physiol 302: F1465–F1477, 2012. First published February 17, 2012; doi:10.1152/ajprenal.00390.2011.—The physiological response to the onset of metabolic acidosis requires pronounced changes in renal gene expression. Adaptations within the proximal convoluted tubule support the increased extraction of plasma glutamine and the increased synthesis and transport of glucose and of NH₄⁺ and HCO₃⁻ ions. Many of these adaptations involve proteins associated with the apical membrane. To quantify the temporal changes in these proteins, proteomic profiling was performed using brush-border membrane vesicles isolated from proximal convoluted tubules (BBMVPCYT) that were purified from normal and acidic rats. This preparation is essentially free of contaminating apical membranes from other renal cortical cells. The analysis identified 298 proteins, 26% of which contained one or more transmembrane domains. Spectral counts were used to assess changes in protein abundance. The onset of acidosis produced a twofold, but transient, increase in the Na⁺/K⁺-dependent glucose transporter and a more gradual, but sustained, increase (3-fold) in the Na⁺/H⁺ exchanger and in the Na⁺/HCO₃⁻ cotransporter. These changes were associated with the loss of glycolytic and gluconeogenic enzymes that are contained in the BBMVPCYT isolated from normal rats. In addition, the levels of γ-glutamyltranspeptidase increased twofold, while transporters that participate in the uptake of neutral amino acids, including glutamine, were decreased. These changes could facilitate the deamination of glutamine within the tubular lumen. Finally, pronounced increases were also observed in the levels of DAB2 (3-fold) and myosin 9 (7-fold), proteins that may participate in endocytosis of apical membrane proteins. Western blot analysis and accurate mass and time analyses were used to validate the spectral counting.

Protein expression in multiple cell types (44). The renal response to metabolic acidosis is characterized by an increased extraction and catabolism of plasma glutamine, an increased reabsorption and de novo synthesis of bicarbonate ions, and an increased synthesis and excretion of ammonium ions that facilitates the excretion of titratable acids (13). This process is initiated in the proximal convoluted tubule, which is the primary site of glutamine extraction and catabolism (60). The adaptive responses within the proximal convoluted tubule are sustained, in part, by increased expression of the genes that encode the mitochondrial glutaminase (15, 69) and glutamate dehydrogenase (68), the cytoplasmic phosphoenolpyruvate carboxykinase (57), and the basolateral glutamine transporter (31) and by activation of the mitochondrial glutamine transporter (56), the apical Na⁺/H⁺ exchanger (52), and the basolateral Na⁺/HCO₃⁻ cotransporter (52). The resulting increases in the corresponding activities facilitate the basolateral uptake of glutamine, the increased reabsorption of bicarbonate ions, the increased synthesis of ammonium and bicarbonate ions, and their vectorial transport across the apical and basolateral membranes, respectively. However, the ultimate excretion of ammonium ions also requires the increased expression of the Na⁺/K⁺-2Cl⁻ cotransporter in the thick ascending limb (30). This adaptation facilitates the removal ammonium ions from the tubular fluid and the creation of a cortical-to-medullary gradient. The ammonium ion gradient provides the driving force for the transport of the appropriate amount of ammonium ions across the α-intercalated cells of the medullary and inner medullary collecting duct (67). The latter process is facilitated by the increased expression of the ammonia channels RhBG and RhCG (6, 7).

While the primary renal adaptations to metabolic acidosis are well characterized, many questions remain regarding the cellular and molecular mechanisms that mediate this response. Genomic and proteomic approaches offer great potential for directing research to answer these questions. For example, microarray analysis (44) compared the mRNAs isolated from whole kidneys of control mice and of mice that were made acidotic for 2 days and 7 days. This analysis detected 13,000 mRNAs or ~40% of the genes on the mouse genomic array. The levels of 333 mRNAs were upregulated and another 342 were downregulated during both acute and chronic acidosis. An even greater number were transiently increased or decreased during onset of acidosis. Cluster analysis indicated that a large proportion of the regulated genes encode solute transporters and proteins involved in cell growth, proliferation, apoptosis, ammoniagenesis, water homeostasis, and energy metabolism. Without prior fractionation, it remains uncertain which of the observed changes occur within a specific cell type or within multiple segments of the nephron. However, this analysis clearly established that the expression of a very large number of genes is altered in the kidney in response to acidosis.

By contrast, mass spectrometry (MS) has been used to profile the proteome of individual nephron segments including the proximal convoluted tubule (16), the thick ascending limb (25), and the inner medullary collecting duct (53, 55). Additional proteomic analyses have been performed with isolated...
intercalated cells (17) and with various established kidney cell lines (24, 34). This approach has also been employed to discover many of the molecular details of the mechanism by which vasopressin activates water (53, 70) and urea transport (28) in the inner medullary collecting duct. An initial proteomic analysis of the temporal changes that occur in isolated proximal convoluted tubules during onset of acidosis was performed using difference gel electrophoresis (16). This analysis identified 21 proteins that are increased and 16 proteins that are decreased. The observed changes indicate that amino acid catabolism and Ca\(^{2+}\) signaling are activated, while conversion of glycine to creatine, oxidation of pyruvate, and fatty acid catabolism is decreased in the proximal convoluted tubule during chronic metabolic acidosis. The temporal analysis also indicated that selective mRNA stabilization may be the primary mechanism by which protein expression is increased or sustained in response to acidosis.

Isolation of subcellular fractions from purified nephron segments offers the potential to characterize the remodeling of the proteome that occurs within specific sites in the cell. Previous studies have established that brush-border membrane vesicles isolated from purified proximal convoluted tubules (BBMV\(_{\text{PCT}}\)) are a highly enriched membrane fraction that is essentially free of contaminating apical membranes from other cells within the renal cortex (16, 66). In the present study, this isolation protocol was used to produce a proteomic profile of the temporal changes that occur in the BBMV\(_{\text{PCT}}\) during onset of acidosis. This analysis identified 298 proteins and characterized novel adaptations in proteins that participate in carbohydrate and amino acid transport and metabolism and in the recycling of proteins to and from the brush-border membrane.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats weighing \(-200\) g (8–10 wk old) were obtained from Charles River Laboratories (Kingston, NY). The rats were allowed free access to a rodent chow (Harlan-Teklad, Madison, WI), and the control group (\(n = 3\)) was given tap water to drink. Arterial blood pH and HCO\(_3\)\(^{-}\) concentration were determined using an i-STAT 1 VetScan (Abaxis). Acute metabolic acidosis (1 day) was induced by stomach loading rats with \(20\) mmol NH\(_4\)Cl/kg body wt and providing \(0.28\) M NH\(_4\)Cl as their drinking water (\(n = 3\)). This protocol produced a pronounced decrease in blood pH (7.11 ± 0.04) and HCO\(_3\)\(^{-}\) concentration (8.8 ± 1.1 mM). Rats that were made acidic for 3 days or 7 days were simply provided with 0.28 M NH\(_4\)Cl as their sole source of drinking water (\(n = 3\) each). After 3 days, this protocol produced a level of acidosis that is similar to the acute treatment (61), but by 7 days the acidosis is partially compensated (pH = 7.34 ± 0.02, (HCO\(_3\)\(^{-}\)) = 15.6 ± 0.9 mM) By contrast, the control rats had an arterial blood pH of 7.37 and metabolism and in the recycling of proteins to and from the brush-border membrane.

**Isolation of brush-border membrane vesicles.** BBMV\(_{\text{PCT}}\) were prepared from isolated PCTs using the standard method of MgCl\(_2\) precipitation (4, 5). Purified tubules were resuspended in 10 vol/wt wt of a solution containing (in mM) 300 mannitol, 5 EGTA, 1 PMSF, 1 sodium orthovanadate, and 12 HEPES, pH 7.1. After polytron treatment (90 s, setting 5), the homogenate was diluted twofold with H\(_2\)O\(_2\) and then MgCl\(_2\) was added to yield a final concentration of 12 mM. The mixture was then incubated on ice for 15 min with intermittent and gentle mixing. Following centrifugation at 3,000 g for 10 min at 4°C to remove mitochondria and cellular debris, the resultant supernatant was centrifuged at 30,000 g for 40 min at 4°C to pellet the BBMV. The pellet was then resuspended in 1 volume of (in mM) 150 mannitol, 2.5 EGTA, and 6 HEPES, pH 7.1 and homogenized with 15 passes of a glass Tenon homogenizer. The BBMV\(_{\text{PCT}}\) were again precipitated by the addition of 12 mM MgCl\(_2\) and repetition of the incubation and centrifugation steps. The final pellet was resuspended in the previous mannitol buffer, and the BBMV\(_{\text{PCT}}\) were stored at \(-80^\circ\)C.

**Sample preparation and mass spectrometric analysis.** Aliquots of BBMV\(_{\text{PCT}}\) containing 100 µg of protein were denatured by heating at 95°C for 5 min. After cooling to room temperature, the samples were dried and reconstituted to 3 mg/ml in 7 M urea/2 M thiourea. The ultrasonicated samples were reduced (14 mM dithiothreitol, 30 min, 37°C), alkylated (7 mM iodoacetamide, 1 h, 37°C), and then diluted fivefold with 100 mM ammonium bicarbonate, pH 8.0. Sequencing grade modified tryspin (Promega) was added (1:30, enzyme/protein), and the samples were incubated for 16 h at 37°C. The peptides were dried (SpeedVac) and reconstituted in 30 µl of 0.1% formic acid/3% acetonitrile. Each sample was analyzed in triplicate by injecting 1.5 µl aliquots onto an Agilent C-18 reverse phase HPLC chip (15 µm ID × 1.5 cm) with a nanospray tip. Peptides were eluted directly into an Agilent 6520 QTOF mass spectrometer using a 12-min linear gradient of 15%–45% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min. MS spectra were collected over an m/z range of 200 to 2,400 kDa. MS/MS spectra were collected from the triplicate injections using a different data-dependent acquisition mode for each injection: 1) the eight most abundant ions in each MS scan were selected for MS/MS analysis with no dynamic exclusion limit, 2) the five most abundant ions in each MS scan were selected using a dynamic exclusion of three MS/MS spectra of a given mass within an exclusion duration of 20 s, and 3) the same conditions as in “2” but including an exclusion list of the 10 most abundant proteins and their respective peptides identified in the first two injections. Compound lists of the resulting spectra were generated using Mass Hunter Acquisition 3.2 software (Agilent) with an intensity threshold of 1,000 and 1 scan/group.

**Bioinformatic analysis.** The resulting mass spectra were searched against the UniProt-KB *Rattus norvegicus* database using SEQUEST and X!Tandem (version 2009.10.01) search engines (11, 21, 72). Each search was performed with a mass tolerance of 50 ppm for MS and 0.1 kDa for MS/MS spectra and with settings for tryptic peptides with up to two missed cleavages, with carbamidomethylation of cysteine as a fixed modification, and with oxidation of methionine as a variable modification. Peptide false discovery rates (FDR) were determined by a target decoy approach using a reversed database concatenated to the parent forward database (19, 29). The results of the searches were combined, and the identifications were validated using Scaffold version 3.06 (Proteome Software). Peptide and protein identifications were validated with settings of 95% Peptide Prophet (32), 80%
Protein Prophet (43), and a minimum precursor mass error of 15 ppm. Lists of protein identifications were assembled separately for each sample group (control, 1 day, 3 days, or 7 days acidic). An additional requirement for protein identification was a minimum of two identified peptides per protein or the manual verification of a protein identified from a single peptide. Only proteins that were identified in a minimum of two biological replicates were included in the final list of identified proteins.

**Spectral counting.** Spectral counting was used to assess the relative abundance of a protein in each sample group (47, 48). Similarity of sampling across injections and biological samples were tested by comparing the spectral counts (SpC) for each injection and each biological sample. SpC for each protein were computed by summing the spectra identified using SEQUEST and X!Tandem from each of the three data acquisition modes per biological sample. Principal components analysis and Pearson’s test for correlation were performed using both the R statistics package (version 2.11.1) (http://www.r-project.org) and the DAnTE version 1.2 software package (http://omics.pnl.gov/software/DAnTE.php) (51) to assess and visualize similarity across all the samples, between injections, and among each of the biological samples. Spectral data were compared for variance by analyzing the number of proteins identified per sample group, the percent of the total spectra identified, and the percent of the total peptides identified. Once the similarity and variance between the biological samples were determined, the significance of the differences in SpC was calculated.

**Cluster analysis and heat maps.** SpC data were normalized to the total spectra in each sample and then filtered to display proteins that were identified in a minimum of three injections per sample group. To prevent calculating logarithms of zero, a pseudocount of one was added to each SpC of the filtered data (3, 45). The resultant normalized data were then analyzed using the hierarchical clustering function in DAnTE using an average agglomerative method with a Spearman distance matrix.

**Statistical analysis.** Significant changes between the sample groups were calculated using the filtered SpC data produced for the cluster analysis. Student’s t-test was used to infer significance of differentially expressed proteins. A P value < 0.05 and a minimum of two identified peptides per protein were used as the criteria for a significant change in protein abundance between the control and each of the acidic groups. For each protein, the log2 ratio of the SpC for the 1 day-, 3 day-, or 7 day-acidic samples versus the control samples was calculated using Eq. 1, where ncontrol and nacidotic are the total SpC in the control and acidotic samples, respectively, and t is the total SpC for all of the identified proteins in each of the two samples (3, 45).

\[
R_{SpC} = \log_2 \left( \frac{n_{acidotic} + 1.0}{n_{control} + 1.0} \right) + \log_2 \left( \frac{t_{control} - n_{control} + 1.0}{t_{acidotic} - n_{acidotic} + 1.0} \right)
\]  

Once significant differences were determined, the ratio of spectral counts (R_{SpC}) values were plotted versus the total SpC for each protein in the combined control and acidotic samples. The corrected P values were then used to visualize the distribution of the proteins that were significantly different in the 1 day-, 3 day-, and 7 day-acidic BBMV sample groups versus the control group.

**Enrichment of functional annotations.** Scaffold files were created separately for each of the control or acidic groups. Lists of total proteins per sample were tested for enrichment of functional annotations using the manually curated Gene Ontology (2) terms within the Scaffold software. Functional annotations were selected based on known inferences of cell function (i.e., brush-border membrane, proximal tubule function, glycosylation, etc.). Lists of interest were assembled based on these inferences and then expanded by manually evaluating proteins whose functional annotations were incomplete.

**Validation of the SpC data.** Accurate mass and time (AMT) tag analyses and Western blot analysis (66) were used to validate some of the results obtained by spectral counting. AMT analysis identifies specific peptides by their accurate mass weights (<10 ppm) and the reproducibility of their time of elution during the initial liquid chromatographic (LC)-MS analysis (50). Due to the reduced complexity of the purified BBMV_{PCT} samples (66), it was feasible to employ AMT analysis to validate many of the conclusions derived from comparison of SpC data. The individual X!Tandem data files for the nine replicates of each treatment were assembled into an AMT database using the createamt command of msInspect version 2.3 (41). LC-MS elution times for each replicate were identified separately using the findpeptides mode of msInspect version 2.3. The profiles were filtered so that only MS profiles with two or more monoisotopic peaks were included in the analysis. The MS peaks in each LC-MS profile were then matched to the AMT database and aligned using the peptidearray function. Finally, the matched features from the replicate datasets were analyzed for changes in peak intensity. The log2 adjusted data were Loess normalized using DAnTER version 0.1.1 (51). To determine significance, the altered abundances were analyzed using ANOVA at both the peptide and protein level. Primary antibodies for NHERF-3 (Transduction Laboratories), aminopeptidase N (Santa Cruz Biotechnology, Santa Cruz, CA), fructose-bisphosphate aldolase B (Acros Antibodies), enolase (Abcam), SGLT2 (Abcam), and glyceraldehyde 3-phosphate dehydrogenase (Imgenex) were obtained from the indicated suppliers and used for Western blot analysis (66). Analyses were performed using isolated BBMV_{PCT} samples except for NHERF3, which was quantified in homogenates of isolated proximal convoluted tubules. Dylight 680 conjugated goat anti-mouse or 400 goat anti-rabbit IgG (Pierce) was used as secondary antibody. The resulting complexes were visualized and quantified using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE).

**Data files.** Raw data files are available for download from Tranche at https://proteomecommons.org/ (20) using the following hash: RoONJnZyWXHfeEFU5Os70V1k7ZJ19uERG7Km5510sJpK0uw-b4c6okINlIgTg+JF672AqTgrcKTTAHId0gY2AXZNAAAAAA-AAmimg==.

**RESULTS**

Proteomic analysis of BBMV_{PCT} from control, 1 day-, 3 day-, and 7 day-acidotic rats. By microscopic inspection, the isolated tubules were ~95% proximal convoluted tubules (S1 and S2 segments) with very few proximal straight tubules (S3 segments). The isolated tubules were homogenized and used to isolate BBMV_{PCT} by MgCl2 precipitation. This protocol yields a 15-fold enrichment of BBM markers compared with homogenates of kidney cortex (66). The BBMV_{PCT} were subjected to in-solution digestion and proteomic analysis using a QTOF MS equipped with a C18-reverse phase HPLC-chip as outlined in Fig. 1. To increase peptide identifications, precursor ions from triplicate injections were selected for collision-induced dissociation using three different dynamic acquisition methods (75). The incorporation of an exclusion list in the third dynamic acquisition mode increased the number of unique peptides and proteins identified. Spectra were identified using both SEQUEST and X!Tandem spectral matching algorithms, which further increased the spectral assignments per protein. The FDR for the spectral assignments was determined by searching a reverse sequence database concatenated to the forward database. The search parameters used in this study produced a peptide FDR < 0.1% and a protein FDR < 1.0%. The total number of peptides and proteins identified in all four sample groups were 1,087 and 298, respectively. Gene Ontology annotations, as edited in Scaffold, were used to identify the functional categories represented in the four sample groups (Fig. 2). The total number of proteins identified per category was...
was similar for each of the sample groups. The largest categories of identified proteins included membrane proteins, enzymes of metabolic processes, hydrolases, and transporters. These annotations are consistent with the physiological function of the brush-border membrane of the proximal convoluted tubule.

Topology predictions (http://www.cbs.dtu.dk/services/TMHMM/) indicated that 80 proteins, or 26% of the total identified, have a predicted transmembrane domain (TMD). Functional categories for the TMD proteins included receptors, peptidases, and transporters. In total, 27 transporters with six or more TMDs were identified. The functional classification of molecular transport also includes several proteins that participate in translocation and membrane insertion of transporters. The search also identified a novel membrane protein, D3ZTX4, which has a single TMD. A search for conserved domains using the protein families database (http://pfam.sanger.ac.uk) indicated D3ZTX4 contained two trefoil domains (PF00088) that are important for exocellular protection and two glycosyl hydrolase domains (PF01055) that are functional motifs used to hydrolyze carbohydrate bonds. On the basis of this sequence homology, the protein was assigned a functional annotation of a O-glycosyl hydrolase (GO:0004553). Thus, the novel protein may participate in the cleavage of di- or trisaccharides prior to transport across the plasma membrane (40).

The measure of correlation between SpC indicated highly correlated biological replicates with an average r value equal to 0.95 (Fig. 3). The samples from the 1 day- and 3 day-acidotic rats produced the greatest correlation (r ≥ 0.98). These two sets of samples were most different from the 7 day-acidotic samples (r ~ 0.85) and the control samples (r < 0.70).

Quantitative analysis of changes in protein abundance in BBMV"PCT during onset of acidosis. ANOVA was used to determine the statistical significance of the changes in SpC for each protein after 1 day, 3 days, and 7 days of acidosis compared with the control group. The data were plotted as the log_{2} adjusted and normalized ratio of SpC versus total SpC and were color coded to indicate significant differences (Fig. 4). The plot illustrates that it is difficult to validate significant changes in low abundance proteins (<10 SpC). However, significant differences become more evident with increasing abundance. Using this approach it was possible to determine significant differences (P < 0.05) for each protein between each of the acidic groups and the control samples.

Since the SpC for each sample group produced highly correlated data sets, SpC could be used to assess changes in relative protein abundance (22, 36). Hierarchical clustering (Fig. 5A) indicated that several proteins clustered into a group that exhibited a transient decrease in abundance during 1 day and 3 days of acidosis, while others decreased rapidly and remained decreased even after 7 days. Fewer proteins exhibited a transient increase during 1 day and 3 days of acidosis or increased rapidly and remained increased for 7 days. To further illustrate the significant differences, the fold change for each protein in each of the acidic groups was calculated relative to the control group and then plotted versus the calculated P value (Fig. 5B). After 1 day of acidosis, 62 of the 298 identified proteins (20.8%) were significantly (P ≤ 0.05) increased or decreased (±1.5-fold). Most of these proteins remain altered in the 3 day-acidotic animals. However, by 7 days of acidosis, nearly half of the initially affected proteins have returned to control levels, while a new set of proteins are now significantly increased or decreased. Each of these analyses indicates that the proteome of the brush-border membrane of the proximal convoluted tubule undergoes a rapid and pronounced remodeling during onset of acidosis. In addition, each of the affected proteins follows one of the limited subsets of temporal patterns.

Using the Gene Ontology annotations and SpC data, several transporters and other membrane proteins were analyzed for changes in protein abundance (Table 1). On the basis of SpC data, two members of the Na+-dependent glucose transporter family, SLC5A2 and SLC5A8, were significantly increased in abundance during onset of acidosis. The apical Na+-dependent glucose transporter SLC5A2 (aka SGLT2) is increased nearly twofold within 1 day and 3 days of onset of acidosis, but returns to normal by 7 days of acidosis. SGLT2 is the primary glucose transporter in the apical membrane of the early proximal convoluted tubule (62). By contrast, the apical Na+-
dependent lactate transporter SLC5A8 (aka SGLT5) (49) is increased more gradually and remains elevated (3-fold) even after 7 days of acidosis. Additional transporters that exhibit less pronounced but significant increases include the apical Na\(^{+}\)/H\(^{+}\)-dependent phosphate transporter SLC34A3 (aka NaPi2C) and the very abundant basolateral Na\(^{+}\)/H\(^{+}\)/3HCO\(_{3}\)/H\(^{+}\) cotransporter SLC4A4 (aka NBC-1). By contrast, the Na\(^{+}\)/H\(^{+}\)-dependent phosphate transporter SLC34A1 (aka NaPi2A) is rapidly decreased during onset of acidosis but returns to normal levels by 3 days of acidosis. The Na\(^{+}\)/K\(^{+}\)-ATPase-α (Na\(^{+}\)/K\(^{+}\)-ATPase subunit α-1) and -β (AT1B1) subunits are primarily localized to the basolateral membrane. The levels of the two Na\(^{+}\)/K\(^{+}\)-ATPase subunits associated with the BBMV\(_{\text{PCT}}\) samples were unchanged during onset of acidosis (data not shown). The B2, C1, E1, F, and L subunits of the V-type H\(^{+}\)-ATPase, a known apical marker, were also identified (Table 1). The B2 subunit is characteristic of the H\(^{+}\)-ATPase that is expressed in the apical membrane of the proximal tubule (65). Of these, the C1 and E1 subunits increased significantly (≥2-fold) by 3 days of acidosis. By contrast, the levels of the B2 and F subunits were decreased slightly.

Proteins identified as having peptidase activity were also quantified (Table 2). On the basis of total SpC, dipeptidyl peptidase IV, aminopeptidase N, X-prolyl aminopeptidase, and γ-glutamyltranspeptidase were the most abundant peptidases. On the basis of SpC data, γ-glutamyltranspeptidase and glutamate carboxypeptidase were increased significantly but only after 7 days of acidosis. However, collectrin (TMM27), a type 1 transmembrane protein that is required for development of cilia and the delivery of the primary neutral amino acid transporter (B\(^{0}\)AT1) to the apical membrane (74), was increased twofold in all of the acidotic samples. By contrast, aminopeptidase N decreased significantly in the 1 day- and 3 day-acidotic groups, and a soluble dipeptidase (CNDP2) was detected only in the control sample.

![Fig. 2. Functional classification of proteins identified in control and acidotic brush-border membrane vesicles isolated from proximal convoluted tubules (BBMV\(_{\text{PCT}}\) samples. The proteins identified in the control, 1 day-, 3 day-, and 7 day-acidotic sample groups were classified into selected Gene Ontology annotations using Scaffold software. To better visualize the data, the protein counts were plotted on a log scale.](http://ajprenal.physiology.org/)

![Fig. 3. Correlation of overall proteomic data obtained from control, 1 day-, 3 day-, and 7 day-acidotic rats. Heat map of Pearson’s correlation data among the 12 biological samples (3 per group). Colors indicate low correlation (r ≈0.65, blue) to highest correlation (r ≈0.99, red).](http://ajprenal.physiology.org/)

![Fig. 4. Statistical analysis of SpC data. SpC were summed for each protein and then plotted vs. the RSC values. Colored data points indicate those proteins that were significantly increased (red) or decreased (green) vs. the control group. To account for the variance among samples, the statistical analysis was calculated using a Student’s t-test (P ≤ 0.05) prior to summing the SpC.](http://ajprenal.physiology.org/)
Our previous analysis established that BBMVPCT contain significant levels of many of the enzymes of glycolysis and gluconeogenesis (66). Most of these proteins are significantly decreased in BBMVPCT isolated from 1 day-, 3 day-, and 7 day-acidotic animals (Table 3). These include fructose-1,6-bisphosphatase, an enzyme that is unique to gluconeogenesis, and five enzymes that catalyze reversible reactions of the glycolytic pathway. Additional enzymes of carbohydrate metabolism that are decreased include transketolase and cytoplasmic isoforms of malate dehydrogenase and isocitrate dehydrogenase. Of this group, only aldolase decreases transiently during 1 day and 3 days of acidosis and then apparently returns to control levels.

Proteins involved in cytoskeleton structure, membrane scaffolding, vesicle trafficking, and signal transduction were also analyzed (Table 3). NHERF1, NHERF3, and PDZK1IP are known apical markers of the proximal tubule (64). This group of proteins contain PDZ, PDZ interacting, Src homology, or plekstrin homology domains that function to target proteins to the apical plasma membrane via their scaffolding interactions. These results, combined with the identification of ezrin, radixin, moesin, and proteins of the actin filament complex are consistent with enrichment of BBMVPCT (35). Of these proteins, only myosin 9 was greatly increased (10-fold), while NHERF3 and DAB2 were significantly decreased (2-fold), during early onset of acidosis. Identified proteins involved in vesicle trafficking included various isoforms of Rab, Ras, and Rho, all of which are important for vesicle trafficking and endosomal recycling. Two guanine nucleotide-binding proteins, the α-2 subunit of Gi and the β-2 subunit of Gβ/γ, were also observed. Both are membrane-associated proteins that are GTP-dependent modulators of signal transduction. Sequence annotation from UniProt (www.uniprot.org) suggests that Gi α-2 subunit may function as a MAPKK activator. Of these proteins, only Rab1A and Rab7A exhibit transient decreases during onset of acidosis. An alphabetic list of all of the identified proteins and their total SpC for each group of samples is available from Tranche at (https://proteomecommons.org/).

Validation of the SpC data. ANOVA analysis of the AMT data set identified several proteins whose abundance was significantly altered compared with the control samples ($P < 0.05$, fold change $>1.5$). Most of these proteins were also identified by comparing changes in SpC data. A comparison of the relative changes in abundance determined by SpC and AMT analysis demonstrates that the results of the two analyses are highly correlated (Fig. 6). Of particular interest were the identified enzymes of glycolysis and gluconeogenesis. For example, AMT analysis confirmed that the levels of fructose 1,6-bisphosphatase-1, enolase, and glyceraldehyde 3-phosphate dehydrogenase were significantly decreased in the BBMVPCT samples during acidosis. However, for this group of enzymes, the decreases observed in SpC analysis were greater...
Table 1. Transports

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<th>Protein</th>
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<th>Mass, kDa</th>
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<th>Control</th>
<th>1 Day</th>
<th>3 Day</th>
<th>7 Day</th>
<th>Functional Subclass</th>
<th>Apical?</th>
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<td>Sodium/sulfate cotransporter, SLSa8</td>
<td>D3Z9E8</td>
<td>66</td>
<td>13</td>
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<td>8.67 ± 1.5</td>
<td>20.33 ± 0.3</td>
<td>16.67 ± 1.9</td>
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<tr>
<td>Na⁺-glucose cotransporter 5, Slc5a10</td>
<td>SLC5A10</td>
<td>12</td>
<td>5</td>
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<td>1.00 ± 0.0</td>
<td>1.00 ± 0.0</td>
<td>8.33 ± 1.5</td>
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<tr>
<td>Sodium- and chloride-dependent creatine transporter 1</td>
<td>SLC6A8</td>
<td>71</td>
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<td>1.00 ± 0.0</td>
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<tr>
<td>Sodium-dependent neutral amino acid transporter B'AT3</td>
<td>SLC3A2</td>
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<tr>
<td>Sodium-dependent neutral amino acid transporter B'AT1</td>
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<td>69</td>
<td>11</td>
<td>13.00 ± 1.5</td>
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<td>15.67 ± 3.8</td>
<td>6.33 ± 0.9</td>
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<tr>
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<tr>
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<tr>
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<td>O89035</td>
<td>31</td>
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<td>4.67 ± 2.3</td>
<td>5.00 ± 1.0</td>
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<tr>
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<td>8.00 ± 2.1</td>
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<td>7.67 ± 1.9</td>
<td>4.67 ± 1.9</td>
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<td>Y</td>
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<tr>
<td>Aquaporin-1</td>
<td>AQP1</td>
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<td>8.00 ± 3.1</td>
<td>8.67 ± 0.9</td>
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<tr>
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<td>V-type proton ATPase subunit B, brain isoform</td>
<td>VBAT2</td>
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<td>40.33 ± 2.4</td>
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<td>23.67 ± 3.2</td>
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<td>12</td>
<td>7.67 ± 1.2</td>
<td>2.00 ± 1.0</td>
<td>2.33 ± 0.7</td>
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<td>Y</td>
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<tr>
<td>V-type proton ATPase 16 kDa proteolipid subunit</td>
<td>VATL</td>
<td>16</td>
<td>4</td>
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<td>12.67 ± 8.8</td>
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<td>Y</td>
</tr>
<tr>
<td>ATPase, H⁺ transporting, lysosomal 38 kDa, V0 subunit d1</td>
<td>Q5M776</td>
<td>40</td>
<td>24</td>
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<td>1.00 ± 0.0</td>
<td>2.00 ± 0.6</td>
<td>7.00 ± 1.2</td>
<td>15</td>
<td>Y</td>
</tr>
<tr>
<td>ATPase, H⁺ transporting, V1 subunit D, isoform CRA_c</td>
<td>Q6P503</td>
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<td>13</td>
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<td>4.33 ± 2.0</td>
<td>15</td>
<td>Y</td>
</tr>
<tr>
<td>ATPase, H transporting, lysosomal V1 subunit GI</td>
<td>B2GIV5</td>
<td>14</td>
<td>2.33 ± 0.3</td>
<td>2.33 ± 0.3</td>
<td>2.33 ± 0.3</td>
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<td>Y</td>
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<tr>
<td>Transitional endoplasmic reticulum ATPase</td>
<td>TERA</td>
<td>89</td>
<td>11.67 ± 0.3</td>
<td>14.33 ± 1.3</td>
<td>11.67 ± 0.3</td>
<td>10.67 ± 0.9</td>
<td>15</td>
<td></td>
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</table>

Values are means ± SE. Y, yes. Mean spectral counts: *P < 0.01 and †P < 0.05 (upregulated); ‡P < 0.01 and §P < 0.05 (downregulated). Functional subclass: 1, dicarboxylic acid; 2, amino acid; 3, neutral amino acid; 4, bicarbonate; 5, carbohydrate; 6, neurotransmitter; 7, creatine; 8, monocarboxylate; 9, organic cation; 10, organic anion; 11, ammonium; 12, urate; 13, phosphate; 14, glycerol/ water; 15, ATP cassette or ATPase; 16, dipeptidase.

than those determined by AMT analysis. As illustrated by the β-subunit, a large fold increase was observed for many of the subunits of the mitochondrial ATP synthase. This observation suggests that during acidosis there may be an increase in the abundance of mitochondria that are positioned in close proximity to the endocytic apparatus immediately underneath the brush-border membrane. By contrast, there is a pronounced decrease in the fatty acid binding protein-1 that is associated with the BBMV_PCT samples during acidosis. This observation is consistent with previous data obtained by two-dimensional gel analysis of proximal convoluted tubules isolated from normal and acidic rats (16). In rodents, this low molecular weight protein is synthesized in the liver, filtered by the glomeruli, and reabsorbed in the proximal convoluted tubule where it is primarily localized in endocytic vesicles. Finally, AMT analysis indicates that the Na⁺-dependent glucose transporter-2 (SGLT-2 or SC5A2) transporter is significantly increased following 3 days of acidosis (data not shown), although a significant change was not detected after 1 day of acidosis as in the SpC data.
Western blot analysis was also used to confirm some of the SpC data (Fig. 6). This approach indicated that the levels of aminopeptidase N were decreased even after 7 days of acidosis. By contrast, Western blot analysis suggested that aminopeptidase N was decreased only in the 7-day-acidotic animals. However, the Western blot analysis data obtained for NHERF3, aldolase B, enolase, and glyceraldehyde 3-dehydrogenase confirmed the previously observed changes. For the latter two enzymes, the decreases quantified by Western blot analysis were in closer agreement with the AMT analysis than the SpC. This probably reflects the lower accuracy of SpC when the total counts are less than 10.

### DISCUSSION

**Proteins involved in carbohydrate transport and metabolism.** During metabolic acidosis, the kidney becomes an important gluconeogenic organ. In rats (46, 54) and humans (23) the α-ketoglutarate generated from the renal catabolism of glutamine is primarily converted to glucose. Previous proteomic analysis established that enzymes of glycolysis and gluconeogenesis are enriched in BBMV_{PCT} compared with BBMV isolated from renal cortex (66). The latter preparation contains apical membranes from multiple cortical cells in addition to the proximal convoluted tubule. The observed enrichment suggests that during normal acid-base balance, enzymes of glucose metabolism may form a complex that is sequestered near or associated with the brush-border membrane of the proximal convoluted tubule. Previous immunofluorescence studies have clearly established that aldolase (38) and fructose 1,6-bisphosphatase (71) are normally localized to the apical membrane of the proximal convoluted tubule (37). Similar observations in erythrocytes suggested that the association of the enzymes of glycolysis with the membrane may generate a higher localized concentration of ATP that is used to support active transport processes (26). The finding that several proteins involved in the reversible steps of glycolysis and in gluconeogenesis are significantly decreased or no longer detectable in BBMV_{PCT} isolated from acidotic rats suggests that the sequestered complex may dissociate from the apical membrane during acidosis. The release of enzymes, including fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase, may support the rapid increase in glucose synthesis (23). The possible return of aldolase to the brush-border membrane following 7 days of acidosis may be due to its functional association with the A, B, and E subunits of the V-type H^+-ATPase and that this interaction is essential for the functional assembly of the H^+-ATPase (39). The transient decrease in aldolase may contribute to the altered ratios of H^+-ATPase subunits that are observed during onset of acidosis.

The observed increase in the Na^+-dependent glucose transporter (SGLT2) was confirmed by Western blot analysis. This finding suggests that a greater proportion of the filtered glucose may be reabsorbed within the early proximal convoluted tubule during onset of acidosis. The reabsorbed glucose could provide energy to support the pronounced remodeling of the cellular proteome that occurs during onset of acidosis. However, given the rapid and pronounced shift toward gluconeogenesis, it is more likely that the reabsorbed glucose undergoes transepithelial transport and is returned to the blood. Finally, the gradual increase in the Na^+-dependent lactate transporter (SGLT5) suggests that lactate may become a more important metabolic fuel...
or gluconeogenic precursor in the proximal convoluted tubule during prolonged metabolic acidosis.

Cytoskeletal, scaffolding, and trafficking proteins. Scaffolding proteins within the apical projections of the proximal convoluted tubule play an important role in the insertion and recycling of brush-border membrane proteins (35). The PDZ domain containing proteins NHERF1 and NHERF3 were quantified in our analysis (Table 3). NHERF1 was originally iden-
tified as a protein kinase A-dependent modulator of the apical Na\(^+\)/H\(^+\)-exchanger (NHE3) activity (73). However, it is now known that NHERF1 interacts with multiple membrane proteins including CLC3, SLC34A1, Gi/Gs/Gt guanine nucleotide binding protein complex, and H\(^+\)-ATPase (35). NHERF1 also interacts with the ezrin-radixin-moesin complex that binds to actin. These interactions contribute to the targeting of vesicles and the insertion of proteins into the apical membrane. Therefore, changes in this class of proteins could contribute to the pronounced remodeling of the brush-border membrane that occurs during onset of acidosis. Several of these proteins were identified in our analysis. However, total SpC detected for many of these proteins were not as pronounced as other classes of proteins. As a result, the observed increase in NHERF1 was not statistically significant. However, the decreases observed for the more abundant NHERF3 and the adapter protein DAB2 were significant. The observed decrease in NHERF3 was confirmed by Western blot analysis. The most significant change was the pronounced increase (10-fold) in myosin 9. The myosin 6 isoform is known to move proteins along actin filaments from the body to the base of the microvilli of the proximal tubule (42). Recent experiments using total internal reflection fluorescence microscopy and a dominant negative form of myosin 6 in opossum kidney cells demonstrated that this isoform participates in the parathyroid hormone-stimulated removal of NaPi2 from the apical membrane (8). Therefore, the observed increase in myosin 9 levels in BBMVP\(_{\text{PCT}}\) isolated from acidotic rats suggests that it may also contribute to the pronounced remodeling of the apical membrane during onset of acidosis.

Rab proteins also participate in the recycling of membrane proteins. We identified two isoforms, Rab5c and Rab7, which are known regulators of endocytosis from apical clathrin coated pits (12, 27). Clathrin levels were significantly increased, while Rab5c and Rab7 were either not detected or transiently decreased, respectively, during 1 day and 3 days of acidosis. Many of the additional Rab proteins have low SpC compared with the proteins that are specific to the apical membrane. These proteins are probably associated with endosomes that are trapped in the vesicles during homogenization.

Amino acid transport and metabolism. The observed increase in \(\gamma\)-glutamyltranspeptidase (\(\gamma\)GT) was confirmed by enzymatic assay (data not shown). This enzyme also catalyzes a phosphate-independent glutaminase activity (14). In normal rats, \(\gamma\)GT is expressed primarily on the luminal surface of the proximal straight tubule (58, 59), which contains little glutamine (60). However, the observed increase in \(\gamma\)GT in BBMVP\(_{\text{PCT}}\) suggests that during acidosis, it may facilitate the deamidation of filtered glutamine within the lumen of the proximal convoluted tubule. This process would be enhanced by the observed decreases in rBAT and B\(^0\)AT1, which are apical neutral amino acid transporters that facilitate glutamine uptake (9).

Relevance to brush-border membrane physiology. Proteomic analyses are typically completed for two purposes, the identification of proteins in a particular sample and the quantification of changes in protein abundance. In this study, statistical analyses of SpC, Western blot analysis, and AMT data were used to provide an assessment of the changes in protein abundance that occur during onset of metabolic acidosis. A small number of proteins exhibit gradual and sustained changes in abundance even after 7 days of acidosis. However, a larger proportion of the identified proteins exhibit transient increases or decreases. The later observation correlates with the fact that the acidosis produced by feeding rats 0.28 M \(\text{NH}_4\)Cl is partially compensated by 7 days. Thus, the observed changes in expression of the latter group of genes may respond directly to changes in blood pH and/or HCO\(_3\)\(^-\) levels. By contrast, the increased renal excretion of ammonium ions and titratable acids is sustained even though the metabolic acidosis is partially compensated. Thus, the expression of the former set of genes, along with the enzymes involved in ammonium ion and HCO\(_3\)\(^-\) synthesis, may be regulated by a different mechanism.

Previous studies have characterized the effect of metabolic acidosis on the expression of only a few of the apical mem-
brane proteins identified in this study. For example, the Na\(^+\)-dependent phosphate transporter (NaPi-2) is decreased ~0.5-fold in rats made acidotic by feeding NH\(_4\)Cl (1). On the basis of SpC, a similar decrease in the level of NaPi-2 was observed in our 1 day-acidotic animals (Table 1). However, in the present study, the levels of NaPi-2 returned to control values after 3 days of acidosis. This difference may be due to the fact that the previous study used larger amounts of NH\(_4\)Cl to sustain the acidosis, whereas the protocol used in the present study results in partial compensation of the acidosis. The onset of acidosis also causes pronounced changes in expression and localization of the V-type H\(^+\)-ATPase in intercalated cells of the distal nephron (65). However, when measured as an N-independent phosphate transporter (NaPi-2) is decreased of acidosis also causes pronounced changes in expression and localization of the V-type H\(^+\)-ATPase in intercalated cells of the distal nephron (65). However, when measured as an N-independent phosphate transporter (NaPi-2) is decreased.


