Proteomic analysis of the adaptive response of rat renal proximal tubules to metabolic acidosis

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1Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado; and 2Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

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Curthoys NP, Taylor L, Hoffert JD, Knepper MA. Proteomic analysis of the adaptive response of rat renal proximal tubules to metabolic acidosis. Am J Physiol Renal Physiol 292: F140–F147, 2007. First published August 8, 2006; doi:10.1152/ajprenal.00217.2006.—Proximal tubules were isolated from control and acidic rats by collagenase digestion and Percoll density gradient centrifugation. Western blot analysis indicated that the tubules were ~95% pure. The samples were analyzed by two-dimensional difference gel electrophoresis (DIGE) and DeCyder software was used to quantify the temporal changes in proteins that exhibit enhanced or reduced expression. The mass-to-charge ratios and the amino acid sequences of the recovered tryptic peptides were determined by MALDI-TOF/TOF mass spectrometry and the proteins were identified using Mascot software. This analysis confirmed the well-characterized adaptive responses in glutaminase (GA), glutamate dehydrogenase (GDH), and phosphoenolpyruvate carboxykinase (PEPCK). This approach also identified 17 previously unrecognized proteins that are increased with ratios of 1.5 to 5.6 and 16 proteins that are decreased with ratios of 0.67 to 0.03 when tubules from 7-day acidic vs. control rats were compared. Some of these changes were confirmed by Western blot analysis. Temporal studies identified proteins that were induced either with rapid kinetics similar to PEPCK or with more gradual profiles similar to GA and GDH. All of the mRNAs that encode the latter proteins contain an AU sequence that is homologous to the pH response element found in GA mRNA. Thus selective mRNA stabilization may be a predominant mechanism by which protein expression is increased in response to acidosis.

difference gel electrophoresis; mass spectrometry; phosphoenolpyruvate carboxykinase; glutaminase; mRNA stabilization

METABOLIC ACIDOSIS IS A COMMON clinical condition that is caused by the overproduction of an acid or the reduced recovery of bicarbonate and is characterized by a decrease in blood pH and bicarbonate concentration (37). In response to acidosis, the kidneys exhibit a complex set of adaptive responses. Within the renal proximal tubule, this process is characterized by a rapid increase in the catabolism of plasma glutamine (40). Within 1 to 3 h, the arterial plasma glutamine concentration is increased twofold (19) and significant renal extraction becomes evident. Net extraction rapidly reaches 30% of the plasma glutamine, a level that exceeds the percentage filtered by the glomeruli. Thus both apical and basolateral transport of glutamine must contribute to uptake by the epithelial cells of the proximal tubule. In addition, mitochondrial transport and catabolism of glutamine are acutely activated (34). The resulting increases in renal synthesis of ammonium and bicarbonate ions partially restore acid-base balance (4). Further responses include a prompt acidification of the urine that results from an acute activation of NHE3, the apical Na+H+ exchanger (30). This process facilitates the rapid removal of cellular ammonium ions (39) and ensures that the bulk of the ammonium ions generated in the proximal tubule is excreted in the urine. Finally, a pH-induced activation of α-ketoglutarate dehydrogenase reduces the intracellular concentrations of α-ketoglutarate and glutamate (29). Thus the increased catabolism of glutamine initially results from a rapid activation of key transport processes, an increased availability of glutamine, and a decreased concentration of the products of the glutaminase (GA) and glutamate dehydrogenase (GDH) reactions.

During chronic metabolic acidosis, the acute decreases in the renal concentrations of glutamate and α-ketoglutarate are partially compensated and the arterial plasma glutamine concentration is decreased to 70% of normal (4). However, the kidney continues to extract more than one-third of the total plasma glutamine (36) in a single pass through this organ. Renal catabolism of glutamine is now sustained by increased expression of various transporters and key enzymes of glutamine metabolism (7). Following onset of acidosis, a rapid induction of phosphoenolpyruvate carboxykinase (PEPCK) gene expression occurs within the S1 and S2 segments of the proximal tubule (11). A more gradual increase in the level of the mitochondrial GA also occurs solely within the proximal convoluted tubule (9, 43). The adaptations in GA and PEPCK levels result from increased rates of synthesis of the proteins (22, 41) that correlate with comparable increases in the levels of their respective mRNAs (6, 20). However, the increase in GA results from the selective stabilization of the GA mRNA (13, 26, 27), whereas the increase in PEPCK activity results primarily from enhanced transcription of the PEPCK gene (15). The activities of the mitochondrial glutaminase transporter (34) and GDH (42), the apical NHE3 (32), the basolateral SN1 glutamine transporter (25), and NBC1, the basolateral Na+-3HCO3 cotransporter (32), are also increased in the proximal tubule during chronic acidosis. The combined adaptations facilitate 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. The onset of acidosis also causes an increased expression of the apical Na+/dicarboxylate cotransporter, NaDC-1, the cytoplasmic citrate lyase, and the mitochondrial aconitase (1). The latter adaptations contribute to the

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increased reabsorption and metabolism of citrate that support
gluconeogenesis and produce HCO₃⁻. Finally, the renal prox-
imal tubule also undergoes an extensive hypertrophy during
chronic metabolic acidosis (28).

Our current understanding of the renal adaptations to meta-
bolic acidosis has been derived from the work of numerous
investigators who have characterized one or more of the
individual responses. However, it is highly probable that the
cumulative studies in this field have uncovered only a small
fraction of the adaptive responses that occur in the proximal
tubule and are essential to maintenance of acid-base balance.

Proteomics and bioinformatics offer an experimental approach
to accurately quantify the altered expression of multiple pro-
teins and to identify commonalities that provide insight into the
signal transduction pathways and molecular mechanisms that
mediate this essential adaptive response. The current study
reports the application of difference gel electrophoresis (DIGE)
to further characterize the temporal changes in multiple pro-
teins that occur within the rat renal proximal tubule during the
development of chronic metabolic acidosis.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats (150–200 g) were obtained
from the National Cancer Institute-Frederick Cancer Research Facility
or purchased from Charles River. The N-hydroxysuccinimide ester
derivatives of Cy2, Cy3, and Cy5 dyes and all other reagents for
two-dimensional (2-D) gel electrophoresis were obtained from GE
Healthcare. Sypro Ruby stain was purchased from Molecular Probes.
Primary antibodies were obtained from Calbiochem, Cayman Che-
rimicals, Lab Vision, Mito Sciences, R&D Systems, Rockland, and
Upstate. The antibodies specific for arginine-glycine amidinotrans-
ferase and dimethylglycine dehydrogenase were obtained from Dr. J.
Van Pilsum (University of Minnesota) and Dr. R. Brandsch (Univer-
sity of Freiburg), respectively. The rabbit anti-rat renal glutaminase
antibody was prepared as described previously (8). Antibody specific
for the GAC isoform of glutaminase was prepared vs. a unique peptide
sequence (LKETVWKYKSPESN) that is contained in the COOH
terminus of the GAC isomor (Macromolecular Resources, Ft. Col-
mins, CO). The secondary antibodies, goat anti-rabbit IgG IRDye800,
and goat-anti mouse IgG AlexaFluor680, were obtained from Invitro-
gen and Rockland, respectively. The Odyssey blocking buffer and the
black washing boxes were purchased from Li-Cor Biosciences. All
other biochemicals were purchased from Sigma.

Isolation of proximal tubules. Rats were made acidotic by stomach
loading with 20 mmol NH₄Cl and then providing 0.28 M NH₄Cl as
their sole source of drinking water for 2 h and for 1, 3, and 7 days.
The protocols used in this study were approved by the university Animal
Use and Care Committee (protocol 93-250A-12). Proximal tubules
were isolated using a modification of a protocol developed to isolate
mouse proximal tubules (10). Briefly, the rat was decapitated, the
kidneys were removed, and the cortex was dissected and then sliced
with a razor blade into 1- to 2-mm chunks. Approximately 0.8 g of
minced cortex was incubated in a 37°C shaker at 140 rpm in 13 ml of
PBS-glucose (135 mM NaCl, 5 mM KCl, 2 mM NaH₂PO₄, 1 mM
MgCl₂, and 5 mM glucose) supplemented with 4 mM glycine, 1 mM
heptonate, 2 mg/ml collagenase, 1 mg/ml BSA, and 0.1 mg/ml
DNase I. After 15 min, the tissue was dispersed by drawing up and
down 10 times with a 25-ml pipette. The released tubules were
decanted and the process was repeated two more times using a 10-ml
pipette to disperse the tissue. The combined tubules were centrifuged
at 100 g for 1 min, washed once, and resuspended with 40 ml of
42.5% Percoll in PBS-glucose. The sample was centrifuged for 35
min at 35,000 g at 4°C in an SS34 rotor. After centrifugation, the band
of tubules that formed about three/fourths of the way down the tube
was removed, diluted twofold with PBS-glucose, and pelleted by
centrifugation at 100 g for 1 min. The resulting proximal tubules were
divided into two samples that were either homogenized in PBS-
glucose containing 0.1 mg/ml PMSF and 1 μg/ml leupeptin or
solubilized in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS
{3-[(cholamidopropyl)dimethylammonio]-1-propanesulfo-
late} and 30 mM Tris, pH 8.8. Homogenates of kidney cortex were
also prepared. The samples were divided into 0.2-ml aliquots and
stored at −80°C. Protein concentrations were determined using the
Bradford assay (3) with bovine serum albumin as the standard. The
protocol produced 5–10 mg of proximal tubule protein from the
kneys of a single rat.

DIGE analysis. DIGE was performed as described previously (18).
Briefly, for single gel analyses, samples containing 50 μg of a
proximal tubule lysate obtained from control or acidic rats were
labeled with Cy3 or Cy5 dye, respectively. The samples analyzed for
increased protein expression were supplemented with 300 μg of
unlabeled protein from an acidotic proximal tubule lysate, whereas the
samples analyzed for decreased proteins were supplemented with 300
μg of a control proximal tubule lysate. Temporal analyses were
performed by merging the images from four separate 2-D gels. Each
gel included a common 50-μg control containing 6.25 μg of each of
duplicate control, 1-day acidotic, 3-day acidotic, and 7-day acidotic
tubules that were labeled with Cy2 dye. The four control samples were
mixed individually with two 50-μg samples that were obtained from
duplicate control, 1-day acidotic, 3-day acidotic, or 7-day acidotic
tubules that had been labeled separately with Cy3 or Cy5 dye. For
either type of analysis, the combined samples were diluted to 450 μl
with rehydration buffer and isoelectric focusing (IPGphar apparatus,
GE Healthcare) was performed using 24-cm, pH 3-10-nl strips. The
initial isoelectric focusing step requires the use of a nonionic deter-
gen. As a result, DIGE analysis is usually not effective for charac-
terization of membrane proteins. After focusing, the strips were
rinsed, reduced with DTT, and treated with iodoacetamide. The
samples were then subjected to SDS-PAGE using 10 or 12% poly-
acrylamide gel. The gels were imaged using a Typhoon scanner (GE
Healthcare) to individually quantify the fluorescence emitted from
each of the Cy dyes. The gels were subsequently fixed in 30%
ethanol/7.5% acetic acid, stained with Sypro Ruby, and reimaged to
detect total protein. The images were merged and analyzed using
Decyder software to identify protein spots that exhibited greater than
1.5-fold increases or 0.67-fold decreases in abundance. Approx-
imately 50–70 spots were picked from each gel using an automated
Spot Handling Workstation (Ettan, GE Healthcare) that also per-
formed the trypsin digestion, extraction, and spotting of peptides on a
MALDI plate. Further analysis was performed with an ABI 4700
MALDI/TOF/TOF mass spectrometer and proteins were identified
using Mascot software to match the spectra to a rat database.
The corresponding mRNA sequences were downloaded from GenBank
and searched for pH response elements (13, 35) and AU-rich elements
(2) using Omiga software.

Western blot analyses. Samples containing 9.2 μg of a rat renal
proximal tubule homogenate were run on 7.5 to 15% SDS-PAGE gels
in a Bio-Rad Mini-Protein 3 electrophoresis unit. Proteins were
transferred to an Immobilon-FL PVDF membrane (Millipore) in a
Bio-Rad Mini Trans-Blot cell. The gel probe for succinate dehydro-
genase was soaked in a 10 mM CAPS, 10% methanol, pH 11 solution
for 30 min before transfer in this buffer. All other gels were trans-
ferred using a 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol,
pH 8.3 transfer buffer. Following transfer, the membranes were
incubated overnight at 4°C in 25 ml of either 20 mM Tris, 150 mM
NaCl, pH 7.5 (TBS) supplemented with 5% (wt/vol) nonfat dry milk
(for rabbit primary antibodies) or Odyssey blocking buffer (for mouse
primary antibodies). Blots were then equilibrated to room temper-
ature, rinsed twice with 15 ml TBS, and then incubated with 5 ml of
primary antibodies for 1–2 h with gentle rocking. The primary
antibodies were diluted as recommended by the supplier in either TBS
containing 1% nonfat dry milk powder and 0.1% Tween (rabbit antibodies) or in Odyssey blocking buffer with 0.1% Tween (mouse antibodies). The blots were rinsed twice in TBS-0.1% Tween (vol/vol) and then washed four times for 5–10 min each. Goat anti-rabbit IgG IRDye800 secondary antibodies were diluted 1:10,000 in TBS containing 1% milk, 0.1% Tween, and 0.05% SDS and goat-anti mouse IgG AlexaFluor680 secondary antibodies were diluted 1:10,000 in Odyssey blocking buffer with 0.1% Tween and 0.05% SDS. Blots were incubated in 5 ml of the appropriate secondary antibody for 1–2 h and then rinsed and washed as above, but in black washing boxes to minimize exposure to light. The final wash was carried out in TBS lacking Tween. The blots were stored at room temperature in the dark, in the final wash solution, until scanned on a Li-Cor Odyssey Infrared Imager.

RESULTS

Proteomic analysis. Before initiation of a proteomic analysis of the differentially expressed proteins, it was necessary to develop a protocol to isolate rat renal proximal tubules. This was accomplished by modifying an existing protocol to isolate mouse renal proximal tubules (10). The final protocol involved incubation of minced rat renal cortex with 2 mg/ml of collagenase to digest the extracellular matrix, followed by Percoll density gradient centrifugation. The resulting proximal tubules migrate well into the gradient, while glomeruli and other tubular segments collect at the top of the Percoll. The purity of the isolated proximal tubules was confirmed by Western blot analyses using antibodies to proteins that are expressed specifically in the proximal tubule (PT), thick ascending limb (TAL), distal tubule (DT), and collecting duct (CD), respectively.

![Fig. 1. Western blot analysis of isolated proximal tubules. Duplicate samples of rat renal cortex or isolated proximal tubules were separated by SDS-PAGE and probed with antibodies to phosphoenolpyruvate carboxykinase (PEPCK), the Na+/K+-2Cl⁻ cotransporter (NKCC2), the thiazide-sensitive NaCl transporter (TSC), and aquaporin-2 (AQP-2) proteins that are expressed specifically in the proximal tubule (PT), thick ascending limb (TAL), distal tubule (DT), and collecting duct (CD), respectively.](Image)

The Na⁺K⁺-2Cl⁻ cotransporter (NKCC2), the thiazide-sensitive NaCl transporter (TSC), and aquaporin-2 (AQP-2) proteins that are expressed specifically in the proximal tubule (PT), thick ascending limb (TAL), distal tubule (DT), and collecting duct (CD), respectively.

![Fig. 2. Difference gel electrophoresis of proximal tubules isolated from control and 7-day acidotic rats. Separate samples of proximal tubules isolated from control (Cy3) and 7-day acidotic (Cy5) rats were labeled and separated by 2-dimensional (2-D) gel electrophoresis. Images were analyzed using DeCyder software. Yellow spots represent proteins that are unchanged, whereas red or green spots represent proteins that are increased or decreased, respectively. The image is representative of that obtained from 4 separate 2-D gels.](Image)

dyes. DeCyder software was used to ratio the light intensities emitted by nearly 2,000 different proteins that are resolved on the gel (Fig. 2). The software also identifies and quantifies the proteins that are enhanced (pseudo-colored as red) or reduced (pseudo-colored as green) in the sample obtained from the acidotic animal. A robotic workstation was then used to pick ~120 spots containing proteins that are differentially expressed, to perform trypsin digestion, and to spot the resulting peptides on a MALDI plate. The mass-to-charge ratios and the amino acid sequences of the recovered peptides were subsequently determined by MALDI-TOF/TOF mass spectrometry. Mascot software was used to compare the resulting data to a rat protein database and to identify the specific proteins.

The DIGE analysis identified PEPCK and GA as the two proteins that exhibit the greatest fold-induction in response to acidosis (Fig. 3). The major bright red spot in this region of the 2-D gel corresponds to a protein that is increased sevenfold in proximal tubules isolated from 7-day acidic rats relative to its level in control tubules. The protein contained in this spot was identified by MALDI-TOF/TOF analysis to be PEPCK. Similarly, the two red spots, that are increased 8.7- and 8.4-fold, were identified as the 68- and 66-kDa subunits of the mitochondrial GA, respectively. The two major orange spots in this field include: isoforms of succinate dehydrogenase (increased 1.8- and 2.7-fold) and a selenium-binding protein (increased 2.2- and 2.3-fold), the GAC isoform of glutaminase (increased 1.8-fold), multiple species of arginine-glycine amidinotransferase (decreased 0.48-, 0.26-, and 0.17-fold), and enolase (decreased 0.38-fold). The pattern of spots identified as arginine-glycine amidinotransferase is characteristic of a protein that is phosphorylated at multiple sites.

In total, the DIGE analysis identified 21 proteins, including 17 previously unrecognized proteins, that increased between 1.5- and 5.6-fold (Table 1) and 16 additional proteins that...
Table 1. Proteins that are potentially increased in proximal tubules of 7-day acidotic rats

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Accession Number</th>
<th>Mito Protein*</th>
<th>Fold-Increase</th>
<th>Mascot Score</th>
</tr>
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<tr>
<td>Aldehyde dehydrogenase (2)†</td>
<td>P11884</td>
<td>yes</td>
<td>1.5/1.6</td>
<td>381</td>
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<td>Acetoacetyl CoA thiolase</td>
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<td>yes</td>
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<td>239</td>
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<td>Transketolase</td>
<td>P50137</td>
<td>no</td>
<td>1.6</td>
<td>238</td>
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<td>Anchorin CII (annexin)</td>
<td>P14668</td>
<td>no</td>
<td>1.7</td>
<td>238</td>
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<tr>
<td>Glycine decarboxylase</td>
<td>Q91W43</td>
<td>yes</td>
<td>1.7</td>
<td>44</td>
</tr>
<tr>
<td>ER transitional ATPase</td>
<td>P46462</td>
<td>no</td>
<td>1.7</td>
<td>213</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase</td>
<td>Q64536</td>
<td>yes</td>
<td>1.7</td>
<td>118</td>
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<tr>
<td>Dimethylglycine dehydrogenase (2)†</td>
<td>Q63342</td>
<td>yes</td>
<td>1.7/1.9</td>
<td>159</td>
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<tr>
<td>PE binding protein</td>
<td>P31044</td>
<td>no</td>
<td>1.8</td>
<td>183</td>
</tr>
<tr>
<td>GAC-glutaminase (60 kDa)</td>
<td>P13264</td>
<td>yes</td>
<td>1.8</td>
<td>47</td>
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<tr>
<td>Succinate dehydrogenase (2)†</td>
<td>Q920L2</td>
<td>yes</td>
<td>1.8/2.7</td>
<td>241</td>
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<td>5-Oxoprolinase</td>
<td>P97608</td>
<td>no</td>
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<td>Aconitase</td>
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<td>yes</td>
<td>2.1</td>
<td>158</td>
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<td>Q63836</td>
<td>no</td>
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<td>93</td>
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<tr>
<td>Glutamate dehydrogenase (2)†</td>
<td>P26443</td>
<td>yes</td>
<td>2.4/3.0</td>
<td>333</td>
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<tr>
<td>Phenylalanine 4-hydroxylase</td>
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<td>yes</td>
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<td>178</td>
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<td>P04905</td>
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<td>5.6</td>
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<tr>
<td>Phosphoeno-pyruvate carboxykinase</td>
<td>P07379</td>
<td>no</td>
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<td>214</td>
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<tr>
<td>KGA-glutaminase, 66 and 68 kDa†</td>
<td>P13264</td>
<td>yes</td>
<td>8.4/8.7</td>
<td>283</td>
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</tbody>
</table>

Proteins in italics were previously shown to be induced during acidosis. *Mitochondrial protein. †Proteins that exist as multiple isoforms.

Argininosuccinate synthetase (0.67-fold), pyruvate carboxylase (0.63-fold), cathypsin B (0.28), and multiple isoforms of arginine-glycine amidonitrase (0.48- to 0.17-fold). Finally, the levels of two plasma fatty acid binding proteins that are extracted by the proximal tubule, plasma retinol binding protein (0.33-fold) and multiple isoforms of α2-microglobulin (0.16- to 0.03-fold), exhibited pronounced decreases (Fig. 4). In addition, calmodulin, an important Ca2⁺-binding regulatory protein, was induced 5.6-fold within 2 h after onset of acidosis and returned to control levels by 7 days of acidosis.

Duplicate samples of proximal tubules isolated from control rats and from rats that were made acidotic for 1, 3, and 7 days were analyzed to determine the temporal pattern of the adaptive responses (Fig. 5A). This analysis confirmed that PEPCK is maximally induced within 1 day, whereas GA and GDH are gradually increased over 7 days (7). By contrast, the multiple isoforms of arginine-glycine amidonitrase exhibit a gradual decrease and approach a new steady state after 3 days of acidosis (Fig. 5B). In total, the adaptive increases for 10 of the identified proteins were determined (Table 3). The induced proteins were grouped into two distinct kinetic profiles. Only PEPCK and anchorin CII were fully induced within 1 day of acidosis. The two genes were not sufficient to identify common promoter elements that may mediate a pH-responsive increase in transcription. The second group exhibited a more gradual induction with kinetics similar to GA. All eight of the corresponding mRNAs and the PEPCK mRNA contain, within their 3'-untranslated regions, one or more sequences in which seven of eight nucleotides are identical to either of the two 8-nucleotide AU sequences that function to stabilize the GA mRNA during acidosis (26, 27). Two of the four sequences within GDH mRNA that satisfy this criteria were previously shown to function as a pH response element (35). In addition, 4 of 11 mRNAs that encode induced proteins, for which kinetic pro-
files were not determined, also contained a putative pH response element. Thus 12 of 21 proteins that are increased during acidosis are encoded by mRNAs that contain sequences that may function as pH-responsive stabilizing elements.

**Western blot analysis.** Multiple samples of proximal tubules were isolated from control and 7-day acidotic rats and analyzed by semiquantitative Western blot analysis (Fig. 6). The analyses confirmed the well-characterized increases in GA, PEPCK, and GDH and verified the previously uncharacterized increases in dimethylglycine dehydrogenase and the GAC isoform of glutaminase and decreases in the plasma retinol binding protein and arginine-glycine amidinotransferase. Analysis of duplicate tubule preparations confirmed that calmodulin is increased 2.6-fold within 2 h after onset of acidosis and then decreases gradually (data not shown). However, the Western analysis failed to confirm the putative increase in succinate dehydrogenase or the decreases in the multiple isoforms of the plasma α₂-microglobulin (Fig. 6).

**DISCUSSION**

Previous experiments determined that the increase in rat renal PEPCK mRNA is initiated within 1 h following onset of acidosis and reaches a maximum within 7 h at a level that is sixfold greater than normal (20). The sixfold induced level of renal PEPCK mRNA is sustained in rats that are made chronically acidic for periods up to 7 days (21). In contrast, the rat renal GA activity increases gradually within the proximal tubule (2-fold within 24 h) and reaches a plateau after 7 days of acidosis that is eightfold greater than normal (9, 43). The adaptive increases in rat renal GDH mRNA levels (24) and activity (42) also occur gradually within the proximal tubule and reach a maximum threefold induction only after 7 days of acidosis (21).

**Table 3. Induction kinetics of proteins that are increased in proximal tubules after 1, 3, and 7 days of acidosis**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Induction Kinetics</th>
<th>Increase After 7 Days</th>
<th>Potential pH RE</th>
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<tr>
<td>Anchorin CII (annexin)</td>
<td>Rapid</td>
<td>1.8</td>
<td>No</td>
</tr>
<tr>
<td>Glycine decarboxylase</td>
<td>Gradual</td>
<td>1.8</td>
<td>Yes</td>
</tr>
<tr>
<td>Dimethylglycine dehydrogenase</td>
<td>Gradual</td>
<td>2.6</td>
<td>Yes</td>
</tr>
<tr>
<td>GAC-glutaminase (60 kDa)</td>
<td>Gradual</td>
<td>2.3</td>
<td>Yes</td>
</tr>
<tr>
<td>Aconitase</td>
<td>Gradual</td>
<td>1.8</td>
<td>Yes</td>
</tr>
<tr>
<td>Succinate dehydrogenase (2)*</td>
<td>Gradual</td>
<td>3.5/4.7</td>
<td>Yes</td>
</tr>
<tr>
<td>Selenium binding protein (2)*</td>
<td>Gradual</td>
<td>2.1/1.7</td>
<td>Yes</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (2)*</td>
<td>Gradual</td>
<td>2.4/2.6</td>
<td>Yes</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>Rapid</td>
<td>5.9</td>
<td>Yes</td>
</tr>
<tr>
<td>KGA-glutaminase - 66 and 68 kDa*</td>
<td>Gradual</td>
<td>5.5/5.3</td>
<td>Yes</td>
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</table>

*Proteins that exist as multiple isoforms.
chronic metabolic acidosis. Thus the combined DIGE analyses produced data that verified both the fold induction and the kinetic profiles of the three proteins that were previously characterized by more extensive biochemical analysis. This finding verifies the ability to isolate highly purified proximal tubule and adds credibility to the proteomic protocol.

Nearly all of the proteins identified in the temporal studies (Table 3) were increased gradually with kinetics similar to GA and GDH. All eight of these proteins are encoded by mRNAs (Table 3) were increased gradually with kinetics similar to GA and GDH. All eight of these proteins are encoded by mRNAs during acidosis. The PEPCK mRNA also contains a similar AU sequence within the loop portion of a highly conserved stem-loop structure that contrib-
utes to the rapid turnover of the PEPCK mRNA (12). More recent studies have established that this sequence also binds ζ-crystallin and that a pH-responsive stabilization may contribute to the maintenance of the fully induced level of PEPCK during chronic acidosis (unpublished data of Hajarnis S, Taylor L, and Curthoys NP). By contrast, of the 21 mRNAs that encode proteins that are apparently increased (Table 1), only the glutathione S-transferase-P1 mRNA contains the consensus sequence (WWWUAUUAUWW, where W is an A or U) for the classical AU-rich element that mediates the rapid decay of mRNAs that encode various cytokines, transcription factors, and immediate-early response proteins (2). Furthermore, only 3 of the 16 mRNAs that encode proteins that are apparently decreased contain a putative pH response element. Therefore, selective mRNA stabilization may be the predominant mechanism by which protein expression is increased in response to acidosis.

An important observation from the initial experiments is the rapid increase in calmodulin protein (Fig. 4). This protein binds Ca^{2+} ions with high affinity and mediates Ca^{2+}-signaling through its ability to interact with and activate multiple downstream signaling molecules (5, 17, 23). Antibodies specific for the rat calmodulin protein confirmed the rapid but transient increase of calmodulin in the proximal tubule samples used in the DIGE experiments. Further verification of this observation would provide the basis to determine whether onset of acidosis triggers a rapid influx of Ca^{2+} ions into the renal proximal tubule and the activation of various downstream signaling molecules. The influx of Ca^{2+} ions into mitochondria activates multiple dehydrogenase reactions of the tricarboxylic acid cycle, including α-ketoglutarate dehydrogenase (14). Such a mechanism could contribute to the acute activation of the mitochondrial catabolism of glutamine that occurs during onset of acidosis and that clearly precedes the significant increases in GA and GDH levels (44).

The MALDI/TOF/TOF analysis identified the five low molecular weight proteins that appear to rapidly decrease following onset of acidosis (Figs. 2 and 4) as multiple isoforms of α_2-microglobulin. This protein belongs to a family of proteins that bind free fatty acids, their CoA derivatives, and various sterols with high affinity (16). Expression of various fatty acid binding proteins correlates with the ability of a cell to catabolize fatty acids (45). However, α_2-microglobulin is expressed primarily in liver and is secreted into the plasma. In rodents, it is more highly expressed in male animals than females (33). The low molecular weight α_2-microglobulin is readily filtered by the glomeruli and constitutes a major urinary protein (MUP-1) that is used by male rodents to mark their territory. Some of this protein is reabsorbed by the proximal tubule where it is largely found in endocytic vesicles. In contrast to the rapid and pronounced decrease in α_2-microglobulin observed by DIGE analysis, Western blot analysis clearly established that the level of the α_2-microglobulin is unchanged during acidosis (Fig. 6). The Western blot analysis also indicated that succinate dehydrogenase was not increased during acidosis. The two spots that were identified as succinate dehydrogenase had excellent peptide coverage (22 and 28 peptides) and Mascot scores (175 and 241), respectively. However, in both cases, the MALDI/TOF/TOF analysis also identified 18 peptides from PEPCK and assigned Mascot scores of 72 and 60, respectively, for this secondary identification. Close exam-
ination of the imaged gel (Fig. 3) indicates a slight lateral streaking that emanates from the large bright red spot that is PEPCK. Thus the failure to completely focus this very abundant protein probably caused the apparent increase in the adjacent spots that are succinate dehydrogenase. These examples illustrate the importance of confirming the results of the DIGE analysis by using a traditional biochemical approach such as Western blotting.

Other potentially important data include the observed increase in pyruvate dehydrogenase kinase and decrease in pyruvate carboxylase that could reduce oxidation of pyruvate and contribute to the cataplerotic activity of PEPCK. Similarly, observed increases in glycine decarboxylase and dimethylglycine dehydrogenase and the decrease in arginine-glycine amido transferase proteins could shunt renal glycine utilization from the synthesis of creatine into the net production of ammonium ions. Multiple protein spots that either increase (Table 1) or decrease (Table 2) in response to acidosis were identified as a mitochondrial aldehyde dehydrogenase. However, Western blot analysis indicated that the level of this protein was not altered (data not shown). Thus the extent of phosphorylation or covalent modification of aldehyde dehydrogenase may be altered during acidosis. Significant increases in enzymes of glutathione (Pi and Mu isoforms of glutathione S-transferases) and phenylalanine (phenylalanine 4-hydroxylase) metabolism were also observed. Finally, cathepsin B is a lysosomal cysteine protease that participates in protein turnover. The observed decrease in this protein may contribute to the decreased rate of protein turnover that, in part, causes the acidosis-associated hypertrophy of the proximal tubule (31). This finding illustrates the fact that it is not possible to discriminate whether the changes observed in the reported proteomic analysis are direct effects of metabolic acidosis or if they are part of the hypertrophic response that is triggered by the acidosis. Thus the reported analysis has identified a number of potential changes in protein levels that could contribute significantly to the adaptive response to metabolic acidosis and/or renal hypertrophy. However, additional experiments will be necessary to confirm and extend the various hypotheses derived from the proteomic analysis.

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REFERENCES