Differential expression and acid-base regulation of glutaminase mRNAs in gluconeogenic LLC-PK₁-FBPase⁺ cells

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Gstraunthaler, Gerhard, Thomas Holcomb, Elisabeth Feifel, Wenlin Liu, Nikolaus Spitaler, and Norman P. Curthoys. Differential expression and acid-base regulation of glutaminase mRNAs in gluconeogenic LLC-PK₁-FBPase⁺ cells. Am. J. Physiol. Renal Physiol. 278: F227–F237, 2000.—LLC-PK₁-FBPase⁺ cells, which are a gluconeogenic substrain of porcine renal LLC-PK₁ cells, exhibit enhanced oxidative metabolism and increased levels of phosphate-dependent glutaminase (PDG) activity. On adaptation to acidic medium (pH 6.9, 9 mM HCO₃⁻), LLC-PK₁-FBPase⁺ cells also exhibit a greater increase in ammonia production and respond with an increase in assayable PDG activity. The changes in PDG mRNA levels were examined by using confluent cells grown on plastic dishes or on permeable membrane inserts. The latter condition increased the rate of differentiation of the LLC-PK₁-FBPase⁺ cells. The levels of the primary porcine PDG mRNAs were analyzed by using probes that are specific for the 4.5-kb PDG mRNA (p2400) or that react equally with both the 4.5- and 5.0-kb PDG mRNAs (p930 and r1500). In confluent dish- and filter-grown LLC-PK₁-FBPase⁺ cells, the predominant 4.5-kb PDG mRNA is increased threefold after 18 h in acidic medium. However, in filter-grown epithelia, which sustain an imposed pH and HCO₃⁻ gradient, this adaptive increase is observed only when acidic medium is applied to both the apical and the basolateral sides of the epithelia. Half-life experiments established that induction of the 4.5-kb PDG mRNA was due to its stabilization. An identical pattern of adaptive increases was observed for the 5.0-kb primary mRNA. In contrast, no adaptive changes were observed in the levels of the 5.0-kb PDG mRNA in either cell culture system. Furthermore, cultures were incubated in low-potassium (0.7 mM) media for 24–72 h to decrease intracellular pH while maintaining normal extracellular pH. LLC-PK₁-FBPase⁺ cells again responded with increased rates of ammonia production and increased levels of the 4.5-kb PDG and PEPCK mRNAs, suggesting that an intracellular acidosis is the initiator of this adaptive response. Because all of the observed responses closely mimic those characterized in vivo, the LLC-PK₁-FBPase⁺ cells represent a valuable tissue culture model to study the molecular mechanisms that regulate renal gene expression in response to changes in acid-base balance.

A major regulatory function of the kidney is to maintain acid-base homeostasis by excreting excess acids or anions primarily in the form of ammonium salts. It is well established that during metabolic acidosis, the rates of renal ammonia production and excretion are increased significantly (45, 46). The proximal convoluted tubule is the major site of both the basal formation of urinary ammonia and the adaptive increase in glutamine catabolism and ammoniagenesis (9, 11, 13, 49).

The initial reactions in the primary pathway of rat renal glutamine catabolism and ammoniagenesis are catalyzed by two mitochondrial enzymes, phosphate-dependent glutaminase (PDG) and glutamate dehydrogenase (GDH) (Fig. 1). Renal extraction of plasma glutamine occurs through peritubular uptake into proximal tubular cells and by apical reabsorption via a Na⁺-dependent transporter. The glutamine is then transported into the mitochondria and deaminated by PDG. The resulting glutamate is deaminated by GDH, thereby generating two ammonium ions and α-ketoglutarate (Fig. 1). However, in various renal cell culture systems, glutamine is first deaminated by PDG and then the ammonium nitrogen of glutamate is primarily transaminated to pyruvate to form alanine and α-ketoglutarate (19, 32, 35). This pathway yields only one ammonium ion per glutamine. In chronic metabolic acidosis, the elevated rat renal extraction and catabolism of glutamine is accomplished, in part, by increasing the levels of the PDG and GDH enzymes in cells of the proximal convoluted tubule (9, 11, 24, 49). Expression of the cytosolic phosphoenolpyruvate carboxykinase (PEPCK), a key regulatory enzyme of gluconeogenesis, is also increased in this nephron segment (7, 24), suggesting a direct link between the ammoniagenic and gluconeogenic pathways in proximal epithelial cells (41, 44, 46).

Over the past decade, significant progress in understanding the regulation of renal glutamine and ammonia metabolism has been achieved through the use of cell and tissue culture techniques (14, 19, 29, 35, 40) and by molecular biological approaches, including the isolation of the genes for the key regulatory enzymes of glutamine metabolism and the establishment of specific molecular probes. In this context, a full-length PDG cDNA was isolated, sequenced, and expressed (43). By utilizing this molecular probe, the kinetics of...
adaptation of rat renal PDG mRNA that occur in response to alterations in acid-base balance were defined (24, 25, 47). It was shown that the increases in both PDG and PEPCK mRNAs (51) precede the increase in assayable enzyme activities in rat renal cortex. Thus the increases in renal PDG and PEPCK activities (7, 9, 11, 27, 49) are due to an increase in their relative rates of synthesis, which correlate with an increase in the levels of their respective mRNAs (24, 25, 42, 47). However, the mechanisms by which acidosis causes increased expression of the two genes differ significantly (24, 25). The onset of acidosis causes a rapid increase in transcription of the PEPCK gene, which in turn accounts for the increased PEPCK mRNA levels. In contrast, PDG mRNA increases without altering its rate of transcription, suggesting that the increase in PDG mRNA results from increased stability of the mRNA (25). Indeed, a pH-responsive instability element in the 3’-untranslated region of rat PDG mRNA and a specific mRNA-binding protein were recently identified (21, 30). Thus transduction of the initial stimuli produced by acidosis may activate divergent, but temporally coordinated, mechanisms to cause the observed increase in specific mRNAs. However, the signals triggering these adaptive changes, as well as the specific molecular downstream events, are unknown. The characterization of this pathway will require a gluconeogenic and pH-responsive renal cell culture system that can serve as an effective model.

We previously isolated LLC-PK1-FBPase+ cells, a gluconeogenic strain of porcine LLC-PK1 renal epithelial cells (16). LLC-PK1-FBPase+ cells exhibit enhanced oxidative metabolism and decreased glycolytic activity (18). Furthermore, LLC-PK1-FBPase+ cells express significant levels of the key gluconeogenic enzymes, fructose-1,6-bisphosphatase (16) and the cytosolic and mitochondrial isoforms of PEPC (22). It was shown that LLC-PK1-FBPase+ cells express high levels of PDG and PEPC mRNAs (29), which are regulated by culture medium pH with kinetics similar to those observed in vivo in the rat kidney (24). In addition, we recently found that the activity and mRNA levels of only the cytosolic isoenzyme of PEPC are increased in response to acidosis in these cells (22).

Previous studies on the regulation of PDG mRNA in LLC-PK1-FBPase+ cells (29) used slot-blot assays and a rat cDNA probe, pGA13, for which the sequence similarity to the porcine PDG mRNA was unknown. Therefore, to explore in more detail the expression of PDG mRNA in LLC-PK1-FBPase+ cells, a homologous porcine PDG cDNA was cloned (termed pGA201) (38) by screening a LLC-PK1-λ-gt11 cDNA library with the rat PDG cDNA, pGA104 (43). Specific probes derived from the porcine PDG cDNA were then used to characterize the expression of multiple PDG mRNAs in LLC-PK1-FBPase+ cells. Two distinct PDG mRNAs, which are 5.0 and 4.5 kb in length, were clearly expressed in subconfluent cells (38).

In the present study, the expression and acid-base regulation of the two primary glutaminase transcripts were characterized in confluent LLC-PK1-FBPase+ monolayer cultures and in epithelia grown on permeable tissue culture inserts to test the potential effect of decreasing the state of differentiation of the cells (17, 20, 32, 48). We observed that only the 4.5-kb PDG mRNA is increased (3-fold) after adaptation to acidic media for 18 h. However, in filter-grown epithelia, this adaptive response occurs only when acidic medium is applied to both the apical and the basolateral sides. Furthermore, experiments using low-potassium medium indicate that a decrease in intracellular pH is the apparent initiator of this adaptive response.

**MATERIALS AND METHODS**

Cell culture. Gluconeogenic LLC-PK1-FBPase+ cells (16, 18, 22) were cultured in DMEM with 5.5 mM D-glucose, 2 mM L-glutamine, and 26 mM NaHCO3 (pH 7.5), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Culture media were prepared from DMEM-base (D-5030, Sigma Chemical, St. Louis, MO) as described previously (19, 22). Acidic medium (pH 6.9) contained 9 mM NaHCO3 and was supplemented with the appropriate amount of NaCl to maintain equivalent osmolality (19). Low-potassium DMEM was prepared from MEM amino acid solution (M-7020, Sigma Chemical) and MEM vitamin solution (M-6895, Sigma Chemical) as basal components, supplemented with glucose, sodium pyruvate, and inorganic salts without KCl. The potassium concentration of medium after addition of 10% fetal bovine serum was determined by flame photometry. Cultures were incubated at 37°C in a 5% CO2-95% air atmosphere. Routinely, cultures were fed three times a week. Experimental cultures were always fed 24 h before adaptation. Confluent monolayers were subcultured (split ratio 1:3) by using 0.25% trypsin and 0.02% EDTA in Ca2+- and Mg2+-free buffered saline.

 Cultures were grown for 10–12 days to produce confluent monolayers in 10-cm plastic tissue culture dishes (Falcon Optilux, no. 3003) by using 10 ml of culture medium or as epithelial cultures on microporous tissue culture inserts (Nunc TC Inserts, A/S Nunc). Filter inserts (3 inserts/experiment) were placed in 10-cm dishes with an excess of basolateral culture media (15 ml) and an apical medium volume of 2.5 ml.

Adaptation protocols and biochemical assays. Cultures were adapted to metabolic acidosis by switching to acidic media (pH 6.9) for the indicated times. Identical protocols were used for low-potassium adaptation. In previous studies it was shown that gradual decreases of culture medium pH from 7.6 to 6.8 resulted in a gradual increase in the ammoniagenic response in LLC-PK1 cells (19) and in intermediate changes in PDG and PEPC mRNA levels in LLC-PK1-
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PBpase

Fig. 2. PDG mRNAs in rat kidney and in LLC-PK1-FBPase\(^{-}\) cells and corresponding cDNA probes used in the present study. Regions of the PDG mRNAs that have been cloned and sequenced from rat kidney and from porcine LLC-PK1-FBPase\(^{-}\) cells are drawn to scale as solid lines. Dashed lines represent approximate lengths of regions that have not been cloned. Sections of cDNAs used as probes are shown below their corresponding mRNAs. Probes are designated by species (r, rat; p, porcine) and by length (in bp). Thus p2400 is a cDNA containing 3'-untranslated sequence that hybridizes specifically to the porcine 5.0-kb PDG mRNA, whereas r1500 and p930 are probes from the coding region of porcine and rat PDG mRNAs, respectively, that hybridize to both the 4.5- and the 5.0-kb PDG mRNAs from LLC-PK1-FBPase\(^{-}\) cells. AUG, start codon; UAA, stop codon; pA, putative polyadenylation site.

FDCase\(^{-}\) cells (29). Therefore, in the present study, culture conditions (pH 6.9, 9 mM HCO\(_3\)) that slightly exceed a physiological metabolic acidosis were used to maximize the response for Northern analysis (see below). PDG enzyme activity was determined in cell homogenates as described elsewhere (18). Ammonia was analyzed enzymatically in culture media samples by established methods (19).

Northern analysis. For each experiment cells were harvested from either a 10-cm dish or three filter inserts. Total RNA was isolated by using the acid guanidinium thiocyanate method as described in detail (22, 31, 38). Formaldehyde-agarose gel electrophoresis, transfer to GeneScreen Plus membranes (NEN; New England Nuclear), hybridization and posthybridization washings of blots were carried out as described previously (22, 23, 31). Blots were exposed by using either a PhosphorImager Screen (Molecular Dynamics) or autoradiographic film (Kodak BioMax MS). Quantitation of mRNA levels was accomplished using a PhosphorImage Analyzer or a Personal Densitometer SI-Scanner (Molecular Dynamics). Exposure times varied from 48 h for films of PDG blots and 10–12 days for films of PDG blots. Sample integrity and equal loading of 20 µg RNA/lane were monitored by staining with ethidium bromide after electrophoresis. In addition, some blots were also stripped of the initial probe and rehybridized with a human β-actin cDNA (Clontech Laboratories) (31). For the half-life determinations, RNA polymerase II-dependent transcription was blocked by adding 65 µM 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) as described in detail (21, 23).

cDNA probes. The glutaminase cDNA probes used for hybridization include sequences that correspond to the coding region of rat PDG mRNA (r1500), the coding region of both porcine PDG mRNAs (p930), and the 3'-untranslated region of the 5.0-kb porcine PDG mRNA (p2400) (Fig. 2). Probe r1500 is an Acc I restriction fragment of pGA104 (43), and p2400 is an Nhe I/Not I restriction fragment of pGA201 (38). The 4.5- and the 5.0-kb PDG mRNAs were separated and purified from total LLC-PK1-FBPase\(^{-}\) RNA by selective cleavage with RNase H and chromatography on oligo(dT)-cellulose (37). A 1.1-kb segment of coding sequence was PCR amplified and sequenced from the two purified mRNAs by using the same set of primers. The two sequences were identical, and they have a 92% identity with a segment (574–1671 bp) of the rat PDG cDNA (43). The PCR product was cloned into pBluescript SK(-) (Stratagene) and the p930 probe was isolated by restriction with Kpn I and EcoR I (C. Curtis and N. P. Curthoys, unpublished data). For probing PEPCK mRNA, a 1.6-kb Bgl II fragment of pPCK-10 (51, 52), which encodes the rat cytosolic PEPCK, was used. The pPCK-10 plasmid was kindly provided by Dr. R. Hanson (Case Western Reserve Univ.).

Statistical analysis of results was performed using the unpaired Student’s t-test.

RESULTS

Enhanced oxidative metabolism and ammoniagenesis of LLC-PK1-FBPase\(^{-}\) cells and their biochemical response to metabolic acidosis. LLC-PK1-FBPase\(^{-}\) cells were selected by growing the parental LLC-PK1 cells in the absence of added glucose (16). The selected cells express the key gluconeogenic enzymes fructose-1,6-bisphosphatase and cytosolic PEPCK (18, 22, 23, 31) and thus exhibit gluconeogenic competence. In addition, LLC-PK1-FBPase\(^{-}\) cells exhibit an increased oxidative metabolism of glutamine (16), another key feature of renal proximal convoluted tubular cells. As shown in Fig. 3, the increased basal catabolism of glutamine is manifested by an increased mitochondrial

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Fig. 3. Absolute mitochondrial volumes (A), PDG enzyme activities (B), and baseline ammonia production rates (C) of wild-type LLC-PK1 (solid bars) and gluconeogenic LLC-PK1-FBPase\(^{-}\) cells (hatched bars). Values are means ± SD of 3–6 experiments. Mitochondrial volume data are taken from Ref. 18. Enzyme activities were determined in cell homogenates and are expressed as µmol·mg protein·24 h\(^{-1}\) at pH 7.5. Ammonia production rates are expressed as µmol·mg protein·24 h\(^{-1}\) at pH 7.5.
volume density (18) that is paralleled by increased basal activity of the mitochondrial phosphate-dependent glutaminase (PDG) and higher baseline rates of ammonia production.

Furthermore, the LLC-PK₁-FBPase cells exhibit a pronounced increase in ammonia generation in response to a decrease in pH and HCO₃⁻ concentration of the culture medium (Fig. 4). This adaptive response was also significantly greater than that observed with LLC-PK₁ cells (19). In addition, LLC-PK₁-FBPase cells respond with an increase in assayable PDG enzyme activity (Fig. 5), which is not observed in LLC-PK₁ wild-type cells (19). Thus the LLC-PK₁-FBPase cells produce an adaptation to treatment with acidic medium that more closely mimics the overall response to metabolic acidosis that is observed in rat kidney.

Northern analysis of acid-mediated induction of PDG mRNA in dish- and filter-grown LLC-PK₁-FBPase cells. The levels of the two primary PDG mRNAs, which are 4.5 and 5.0 kb in length (38), were analyzed by using the porcine and rat cDNA probes, shown schematically in Fig. 2. The 2.4-kb porcine probe (p2400) encodes the unique 3'-untranslated region of the 5.0-kb mRNA, whereas the 930-bp porcine cDNA (p930) contains a segment of coding sequence that is identical in the 5.0- and the 4.5-kb porcine PDG mRNAs. Thus the former probe is specific for the 5.0-kb PDG mRNA, whereas the latter reacts equally with both PDG mRNAs. A 1.5-kb rat cDNA probe (r1500), which corresponds to the coding region of rat renal PDG mRNA, was also used (Fig. 2, top). The mRNA levels of cytosolic PEPCK were probed with a specific rat cDNA probe from pPCK-10 (22, 51, 52).

![Fig. 4. Rates of ammonia production by wild-type LLC-PK₁ (solid bars) and gluconeogenic LLC-PK₁-FBPase+ cells (hatched bars) in response to treatment with acidic medium (24–72 h at pH 6.9). Values are means ± SD of 3–6 series of experiments. Confluent cultures were adapted for 3 consecutive 24 h-intervals in acidic media. After each incubation interval, culture media were removed and analyzed, and cultures were refed for further incubation. Metabolic rates are expressed as μmol·mg protein⁻¹·24 h⁻¹. *P < 0.05 and **P < 0.01 compared with control (pH 7.5).](http://ajprenal.physiology.org/)

![Fig. 5. Time course of induction of assayable PDG enzyme activity in LLC-PK₁-FBPase+ cells during adaptation to metabolic acidosis. Inset: lack of PDG induction in LLC-PK₁ cells (data taken from Ref. 19). Values are means ± SE with numbers of determinations in parentheses. PDG activity is expressed as mU/mg protein (nmol·min⁻¹·mg cell protein⁻¹). *P < 0.05, statistically significant increases in enzyme activity compared with control values.](http://ajprenal.physiology.org/)

Basal and acid-induced expression of PDG and PEPCK mRNAs were examined in LLC-PK₁-FBPase+ cells grown for 10–12 days to achieve full differentiation on plastic tissue culture dishes or in epithelial cultures on permeable supports. The latter culture condition was developed to further increase the state of differentiation of the polarized epithelial cells (17, 20), which might augment the adaptive response to metabolic acidosis. Phase-contrast microscopic examination revealed remarkable differences in cell shape and cell density of filter-grown LLC-PK₁-FBPase+ epithelia compared with dish-grown monolayers (Fig. 6). In addition, acidic media can be applied to both sides of the filter inserts carrying the epithelial layer or to either the apical or the basolateral side. In the latter cases, a pH and HCO₃⁻ gradient is imposed across the epithelia so
that the sidedness of an extracellular signal or receptor that potentially mediates the pH-response could be determined.

Filter-grown cultures were incubated in control (pH 7.5) or acidic (pH 6.9) media on both sides, or under conditions with acidic media on either epithelial side (apical pH 6.9 or basolateral pH 6.9). After 18 h of incubation, the pH of the media was determined (Fig. 7). The pH of media samples incubated without cells remained constant. Maturation of the cultured epithelium and functional integrity during the adaptation period was monitored by measuring the transepithelial potential difference (17, 20). Under control and acidic conditions (pH 7.5 or 6.9 on both sides), LLC-PK₁-FBPase⁺ epithelia acidify the apical compartment, thereby generating a transepithelial pH gradient of ~0.15–0.20 pH units (Fig. 7). This effect is slightly more pronounced under acidic conditions (Fig. 7, right bars). When incubated with acidic medium on either side, LLC-PK₁-FBPase⁺ epithelia are able to sustain the imposed pH gradient for at least 18–24 h (Fig. 7).

Representative Northern blots of RNA isolated from acid-adapted dish- and filter-grown LLC-PK₁-FBPase⁺ epithelial cells are displayed in Fig. 8. The results of four to six independent experiments are summarized in Fig. 9. Data from acidic cultures were normalized to the respective controls. The 4.5-kb PDG mRNA (probed with p1500 and p930) and the 2.7-kb cytosolic PEPCK mRNA are increased approximately threefold on adaptation to acidic medium for 18 h in LLC-PK₁-FBPase⁺ monolayer cells in tissue culture dishes (Fig. 8, 2nd lane from the left, and Fig. 9, 2nd bar from the left, respectively). In filter-grown LLC-PK₁-FBPase⁺ epithelia, however, increases in PDG and PEPCK mRNAs were only observed when acidic medium was applied to both the apical and the basolateral sides (Fig. 8, right lanes; Fig. 9, right bars; apical + basolateral, pH 6.9). Application of acidic media to either side of the cultured epithelium (apical pH 6.9 or basolateral pH 6.9) was not sufficient to induce a significant response. The levels of cytosolic PEPCK mRNA are somewhat lower in filter-grown LLC-PK₁-FBPase⁺ cells compared with dish-grown cells (Fig. 8, pPCK). However, the acid-mediated increase, normalized to control filter epithelia, was of the same magnitude as found in dishes (Fig. 9C). The 5.0-kb PDG mRNA, which hybridized specifically to the p2400 probe (see Fig. 2), was unchanged and remained at a constant level under all experimental conditions (Fig. 8, top).

Determination of the half-lives of the 4.5-kb PDG mRNA in control and acidic LLC-PK₁-FBPase⁺ cells. In rat kidney, the in vivo induction of PDG mRNA during metabolic acidosis is due to an increased stability of the mRNA (21, 25, 47). To test whether the same mechanism occurs in acid-adapted LLC-PK₁-FBPase⁺ cells, the apparent half-lives of basal and aci-induced 4.5-kb PDG mRNAs were determined by quantitating the rates of decrease that occur after inhibition of RNA polymerase II-dependent transcription with DRB (Fig. 10). Both the control and acid-induced 4.5-kb mRNA bands exhibit first-order decay profiles. However, the apparent half-lives calculated from the rates of decrease were 3.4 h for control and 7.8 h for the acid-induced 4.5-kb PDG mRNA. Thus the apparent half-life of the 4.5-kb mRNA is increased 2.3-fold.

Effects of low-potassium-containing media on ammonia production and on PDG and PEPCK mRNA levels. Potassium deficiency causes an extracellular alkalosis and an intracellular acidosis (1, 5). It also elicits
proximal tubular adaptations similar to those seen in metabolic acidosis (36, 50). On the basis of these observations, it was hypothesized that the proximal tubular adaptations are initiated by changes in intracellular pH (45, 46). Confluent LLC-PK1-FBPase mono-layers were adapted to low-potassium media for 24–72 h to induce an intracellular acidosis at normal extracellular pH. The potassium concentration of the medium was 0.7 mM as determined by flame photometry, compared with 5.4 mM in control DMEM. However, the pH of both media was 7.5. As presented in Fig. 11, LLC-PK1-FBPase cells grown in low-potassium media exhibit an adaptive increase in the rate of ammonia production that is slightly greater after 48–72 h than that observed with acidic medium (for comparison, see Fig. 4). Representative Northern blots are depicted in Fig. 12 and are summarized in Fig. 13. LLC-PK1-FBPase cells also responded to incubation in low-potassium media with a threefold increase in the levels of the 4.5-kb PDG and cytosolic PEPCK mRNAs as occurs on incubation of cells in acidic media. In contrast to using acidic media, where maximal adaptation occurs after 18–24 h (Figs. 8 and 9) (38), the maximal response to low-potassium medium is achieved after 48 h of incubation (Figs. 12 and 13), which is consistent with the sustained increase in ammonia generation (Fig. 11).

**DISCUSSION**

In the present study, confluent cultures of the gluconeogenic LLC-PK1-FBPase cells were tested as an in vitro model system for investigating the molecular mechanisms by which alterations in acid-base balance affect the levels of mRNAs that encode key regulatory enzymes of renal proximal convoluted tubular gluconeogenesis and ammoniagenesis.
In vivo, the adaptive increases in gluconeogenesis and ammoniagenesis in the renal proximal convoluted tubule of carnivorous and omnivorous mammals during metabolic acidosis are coordinated to produce an increased extraction and catabolism of plasma glutamine and an enhanced excretion of titratable acids. Renal catabolism of glutamine leads to the generation of ammonium ions and \( \alpha \)-ketoglutarate (Fig. 1) (10). For net acid secretion to occur, the \( \alpha \)-ketoglutarate, which is a divalent anion, must be neutralized by either complete oxidation or conversion to glucose (44). The flux of \( \alpha \)-ketoglutarate through either pathway also results in the net production and the basolateral release of 2 mol HCO\(_3\)\(^-\), which partially compensates for the systemic acidosis (44–46).

Compared with the parental LLC-PK\(_1\) cells, the gluconeogenic LLC-PK\(_1\)-FBPase\(^+\) cells exhibit an increased oxidative metabolism (18) and an increased rate of glutamine consumption (16). As depicted in Fig. 3, the LLC-PK\(_1\)-FBPase\(^+\) cells also exhibit a greater rate of ammonium ion production that correlates with an elevated basal PDG activity. Furthermore, the LLC-PK\(_1\)-FBPase\(^+\) cells respond to acidification of the culture medium with a pronounced increase in ammonium ion production (Fig. 4) that again correlates with a similar increase in assayable glutaminase enzyme activity (Fig. 5). Although other proximal tubule-like renal cell lines, such as the parental LLC-PK\(_1\) cells and opossum kidney cells, also exhibit slight
increases in glutamine metabolism after exposure to acidic medium, such cells are primarily glycolytic and catabolize glutamine at significantly lower rates (15, 19). Furthermore, LLC-PK_1 wild-type cells lack any adaptive increase in glutaminase activity (Fig. 5, inset; Ref. 19).

During the past decade, renal epithelial and tissue cultures have emerged as a powerful tool to study in vitro several aspects of renal metabolism, epithelial transport, and renal cell growth and differentiation (14, 17). Modern cell and tissue culture techniques enable renal epithelial cells to grow and be maintained at a state of differentiation, comparable with the in vivo tissue. The use of cell biological, immunological, and molecular biological methods has opened new avenues in physiological and pharmacological research. Thus the advantages of using cultured cells in in vitro studies of renal metabolism are obvious and manifold. Easy manipulation of the cells and the ability to change tissue culture parameters individually, in combination, or sequentially in short- or long-term applications have established cultured renal epithelia as valuable tools for investigating renal cell metabolism and function at the cellular and subcellular level. Such studies can also be accomplished without inducing higher ordered regulatory mechanisms as in complex organisms.

Continuous renal epithelial cell lines as well as renal proximal tubular primary cultures from a variety of mammalian species including human have been used to study renal gluconeogenesis and ammoniagenesis in vitro (14, 15, 19, 22, 29, 35). Most of the proximal tubular primary cultures were established to study renal proximal tubular transport functions and hormone responsiveness. Some of the primary cultures were also initiated to study in vitro proximal tubular metabolic features, such as gluconeogenesis, pH-mediated ammoniagenesis, and the expression and regulation of PEPCK and PDG. However, these efforts met with limited success.

In the present study, experiments were performed on 10–12 day confluent LLC-PK_1-FBPase cells and on epithelia grown on permeable tissue culture inserts. Cultured renal epithelia differentiate more when grown on a microporous support because nutrients, hormones, and other factors readily gain access to the basal surface of the epithelium (14, 17, 20, 48). This is well documented in Fig. 6. LLC-PK_1-FBPase cells grown on porous supports form an epithelial layer of differentiated cells with columnar appearance and a significantly higher cell density compared with monolayer cultures grown on plastic. LLC-PK_1-FBPase epithelia exhibit a transepithelial apical negative potential difference of ~1.5 mV and a transepithelial resistance of ~150 Ω/cm² (17, 20). Furthermore, the cultured epithelium generates a transepithelial pH gradient by apical proton secretion and is able to maintain pH gradients that are imposed by adding acidic media on either side of the filter insert (Fig. 7). All of these parameters are strong indicators of the integrity, the transport activity, and the barrier function of LLC-PK_1-FBPase epithelia under the applied culture conditions.

In LLC-PK_1-FBPase cells, two primary PDG mRNAs of 5.0 and 4.5 kb in size are present in subconfluent and confluent cultures (38). The same RNAs could also be readily detected in the present study in confluent dish- and filter-grown LLC-PK_1-FBPase epithelia (Figs. 8 and 12). Specific detection of the two PDG mRNAs was achieved by using separate cDNA probes (Fig. 2). The porcine cDNA probe, p2400, which is derived from the 3′-untranslated region of pGA201, hybridizes specifically to the 5.0-kb PDG mRNA, whereas the rat and porcine cDNA probes r1500 and p930, respectively, contain segments of the coding region of the renal PDG mRNA and hybridize to both the 5.0- and the 4.5-kb mRNAs. The homologous porcine p930 cDNA probe produced stronger signals than the rat r1500 cDNA.

The 5.0- and 4.5-kb PDG mRNAs differ substantially in their response to alterations of the extracellular medium. Only the levels of the 4.5-kb PDG mRNA are increased when LLC-PK_1-FBPase cultures are incubated with acidic (Fig. 8) or low-potassium-containing media (Fig. 12). The 5.0-kb mRNA species appears to be constitutively expressed because its cellular levels were unaltered under all experimental conditions tested. Although the 5.0- and the 4.5-kb PDG mRNAs share an identical stretch of coding sequence (Fig. 2), they clearly contain different 3′-untranslated regions (38). The 3′-untranslated region of the 5.0-kb porcine PDG mRNA lacks the eight-base, AU-rich sequence that was identified as the rat PDG mRNA pH-response element (21, 30, 38). A 48-kDa cytosolic protein from rat kidney cortex binds specifically to the AU repeats and thereby mediates the pH-responsive stabilization of the PDG mRNA (30). LLC-PK_1-FBPase cells also contain a protein that binds specifically to this sequence (O. Laterza and N. P. Curthoys, unpublished data). Thus, from the data presented here, one would predict that the 3′-untranslated region of the 4.5-kb PDG mRNA probably contains a pH-response element that has a sequence that is highly homologous to that of the rat PDG mRNA. The essential function of AU-rich elements in the 3′-untranslated regions of eukaryotic mRNAs and their importance in mRNA stability and turnover have been emphasized in recent reviews (6, 8, 39).

The apparent half-life of the 4.5-kb PDG mRNA is increased 2.3-fold when the LLC-PK_1-FBPase cells were transferred to acidic medium (Fig. 10). This is consistent with the observed 2.5- to 3-fold increase in this mRNA. Thus the level of the 4.5-kb PDG mRNA in LLC-PK_1-FBPase cells is regulated by extracellular pH in a manner identical to that established for the PDG mRNA in rat kidney proximal tubular cells in vivo (9, 10, 24, 25, 41, 42). Furthermore, as seen in vivo (7, 22, 24, 25, 42), the adaptive increase in the level of cytosolic PEPCK mRNA in LLC-PK_1-FBPase cells is mediated by an increased rate of transcription and not by changes in the rate of turnover of the PEPCK mRNA (23, 31).
By culturing LLC-PK₁-FBPase⁺ epithelia on microporous filter inserts, the potential sidedness of the signal that initiates the pH response could be studied (26). As shown in Figs. 8 and 9, both epithelial surfaces must be acidified to elicit a maximal increase in PDG and cytosolic PEPCK mRNAs. This is consistent with what occurs in vivo during metabolic acidosis, where basolateral pH is lowered by the acidic interstitium and apical pH is lowered by the decreased filtered load of HCO₃⁻ and by activation of the Na⁺/H⁺ antiporter. However, in vitro studies of ammonia production by using isolated perfused mouse proximal tubule segments produced different results (33, 34). When the peritubular pH was acutely lowered by decreasing the HCO₃⁻ concentration of the bath buffer, ammonia production increased by 50% (34). However, no increase in ammonia production was observed in the perfused proximal tubules when only the luminal perfusion pH was lowered (34). The different effects observed with isolated perfused proximal tubules and cultured renal epithelium may reflect a difference in how the intracellular pH is affected in the two systems. Alpern and Chambers (3) have clearly demonstrated that with isolated perfused segments a reduction in basolateral pH produces a greater fall in intracellular pH than the same reduction in luminal pH. In contrast, studies with LLC-PK₁ cells grown in plastic dishes and on microporous inserts have shown that changes in extracellular pH produce similar changes in intracellular pH over the range of pH 6.8 to 7.6 (28, 40). Therefore, the cumulative data suggest that the pH-responsive inductions of the PDG and PEPCK genes are not initiated by an asymmetrically distributed membrane receptor that senses changes in the extracellular concentration of either H⁺ or HCO₃⁻ ions. Instead, the observed responses are likely to be initiated in response to a decrease in intracellular pH.

To further test this hypothesis, experimental conditions were selected where the intracellular pH is decreased while normal extracellular pH is maintained. This was accomplished by adapting cultured cells to low-potassium-containing media (Figs. 11 and 12). Potassium depletion leads to cell acidification (1). A low intracellular pH would enhance luminal H⁺ secretion through activation of the luminal Na⁺/H⁺ exchanger and cause an enhanced HCO₃⁻ reabsorption (5). Indeed, chronic hypokalemia does increase the activity of the renal proximal tubule apical membrane Na⁺/H⁺ exchanger, encoded by NHE3 (2, 4). This response was recently reproduced in an in vitro cell culture system (5). Thus the observations in the present study that the low-potassium medium increases the rates of ammonia production (Fig. 11) and produces an increase in both the 4.5-kb PDG and cytosolic PEPCK mRNAs (Figs. 12 and 13) that closely approximate the responses observed with acidic medium strongly support the hypothesis that enhanced ammoniagenesis and increased expression of the two gene products are initiated by a decrease in intracellular pH.

In summary, LLC-PK₁-FBPase⁺ cells, a gluconeogenic renal epithelial cell strain, respond to acidic medium (pH 6.9, 9 mM HCO₃⁻) with an increase in transcription of the cytosolic PEPCK mRNA and a pronounced stabilization of the 4.5-kb PDG mRNA. The inability of the parental LLC-PK₁ cells to exhibit the latter response could be due to a variety of reasons. For example, LLC-PK₁ cells may not express the mRNA-binding protein or they may lack the necessary signaling mechanism that senses changes in intracellular pH and initiates the enhanced interaction necessary to stabilize this variant of the PDG mRNA. The observation that the in vivo response to metabolic acidosis is reproduced in LLC-PK₁-FBPase⁺ cultures in vitro strongly indicates that increased expression of the two gene products is not mediated by a circulating humoral factor. When LLC-PK₁-FBPase⁺ epithelia are grown on permeable filter inserts, both the apical and the basolateral sides must be acidified to elicit the full adaptive response. This observation and the finding that low-potassium medium elicits an identical response suggest that the adaptive response is initiated by a decrease in intracellular pH. Therefore, the renal cells must possess a biochemical mechanism for directly sensing changes in intracellular pH and a pathway to transduce this information into a signal that alters the expression of the two enzymes. Thus the LLC-PK₁-FBPase⁺ strain is a pH-responsive permanent renal cell line that should prove valuable as a tissue culture model to further characterize how renal proximal tubular cells sense pH and how this signal is transduced to increase nuclear transcription and cytosolic mRNA stabilization of specific gene products during metabolic acidosis (12).

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REFERENCES


