pH-responsive, gluconeogenic renal epithelial LLC-PK₁-FBPase⁺ cells: a versatile in vitro model to study renal proximal tubule metabolism and function

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Curthoys NP, Gstraunthaler G. pH-responsive, gluconeogenic renal epithelial LLC-PK₁-FBPase⁺ cells: a versatile in vitro model to study renal proximal tubule metabolism and function. Am J Physiol Renal Physiol 307: F1–F11, 2014. First published May 7, 2014; doi:10.1152/ajprenal.00067.2014.—Ammoniagenesis and gluconeogenesis are prominent metabolic features of the renal proximal convoluted tubule that contribute to maintenance of systemic acid-base homeostasis. Molecular analysis of the mechanisms that mediate the coordinate regulation of the two pathways required development of a cell line that recapitulates these features in vitro. By adapting porcine renal epithelial LLC-PK₁ cells to essentially glucose-free medium, a gluconeogenic subline, termed LLC-PK₁-FBPase⁺ cells, was isolated. LLC-PK₁-FBPase⁺ cells grow in the absence of hexoses and pentoses and exhibit enhanced oxidative metabolism and increased levels of phosphate-dependent glutaminase. The cells also express significant levels of the key gluconeogenic enzymes, fructose-1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK). Thus the altered phenotype of LLC-PK₁-FBPase⁺ cells is pleiotropic. Most importantly, when transferred to medium that mimics a pronounced metabolic acidosis (9 mM HCO₃⁻, pH 6.9), the LLC-PK₁-FBPase⁺ cells exhibit a gradual increase in NH₄⁺ ion production, accompanied by increases in glutaminase and cytosolic PEPCK mRNA levels and proteins. Therefore, the LLC-PK₁-FBPase⁺ cells retained in culture many of the metabolic pathways and pH-responsive adaptations characteristic of renal proximal tubules. The molecular mechanisms that mediate enhanced expression of the glutaminase and PEPCK in LLC-PK₁-FBPase⁺ cells have been extensively reviewed. The present review describes novel properties of this unique cell line and summarizes the molecular mechanisms that have been defined more recently using LLC-PK₁-FBPase⁺ cells to model the renal proximal tubule. It also identifies future studies that could be performed using these cells.

proximal tubule; ammoniagenesis; gluconeogenesis; pH-responsive; acid-base balance

Relationship Between Renal Ammoniagenesis and Gluconeogenesis

THE ONSET OF METABOLIC ACIDOSIS causes a rapid and coordinate increase in the catabolism of plasma glutamine within the renal proximal convoluted tubule (10,99). This adaptation results in the net production of two NH₄⁺ and two HCO₃⁻ ions per glutamine (Fig. 1). The NH₄⁺ ions are largely excreted in the urine, while the newly synthesized HCO₃⁻ ions, along with the bicarbonate reabsorbed from the tubular lumen, are translocated across the basolateral membrane and added to the renal venous blood. In rats and in humans, the remaining carbons derived from glutamine are primarily converted to glucose (19). Thus the well-coordinated increases in ammoniagenesis, bicarbonate synthesis, and gluconeogenesis result in an enhanced excretion of acid equivalents and the partial restoration of acid-base balance. In addition, during metabolic acidosis, the kidney becomes an important gluconeogenic organ that rivals the liver in importance for sustaining glucose homeostasis (14, 93).

During metabolic acidosis, the arterial-venous difference for glutamine across the kidney increases to about one-third of the arterial glutamine (91). Approximately 20% of the plasma glutamine is filtered by the glomeruli and is nearly quantitatively reabsorbed from the lumen of the proximal convoluted tubule (88) by B₀AT₁ (SLC6A19), a Na⁺-dependent neutral amino acid cotransporter (81). Additional glutamine enters the proximal convoluted tubule through the basolateral membrane. The latter uptake is accomplished by reversal of LAT (SLC7A8)-4F2hc (SLC3A2), a heterodimeric neutral amino acid exchanger (61), and/or by the increased expression of SNAT3 (SLC38A3) that catalyzes the reversible Na⁺-depen-
Fig. 1. Renal proximal tubular catabolism of glutamine. During chronic acidosis, approximately one-third of the arterial glutamine is removed during a single pass through the kidney. The glutamine filtered by the glomeruli is nearly quantitatively extracted from the lumen of the proximal convoluted tubule by B^0AT1, a Na^+/H^+ dependent neutral amino acid cotransporter in the apical membrane. Uptake of glutamine through the basolateral membrane occurs by reversal of the neutral amino acid cotransporter (LAT2) and/or through increased expression of the basolateral glutamine transporter (SNAT3). Increased renal catabolism of glutamine is facilitated by increased expression (arrows) of the genes that encode glutaminase (GA), glutamate dehydrogenase (GDH), phosphoenolpyruvate carboxykinase (PEPCK), the apical Na^+/H^+ exchanger (NHE3), and SNAT3. In addition, the activities of the mitochondrial glutamine transporter and basolateral Na^+/HCO_3^- are increased (+). Increased expression of NHE3 contributes to the transport of ammonium ions and the acidification of the luminal fluid. The combined increases in renal ammonium ion excretion and gluconeogenesis result in a net synthesis of HCO_3^- ions that are transported across the basolateral membrane by the Na^+/HCO_3^- cotransporter (NBC1). αKG, α-ketoglutarate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate.

Parental Porcine Renal Epithelial LLC-PK1 Cells

The LLC-PK1 cell line (ATCC CL-101) was developed in 1958 from a mince of the whole kidney of a normal male Hampshire pig (Sus scrofa) (43). The cells retain several features of the proximal tubule (31). LLC-PK1 cells are epithelial and nontumorigenic, form domes, and produce considerable amounts of plasminogen activator. The cells also express high activities of renal brush-border membrane marker enzymes, Na^+-dependent apical transport of glucose, amino acids, and phosphate, and a basolateral, secretory transport of organic cations. Morphologically, the apical membrane domain is characterized by the presence of microvilli, which increase in number and size with time in culture (74). Cell-cell contacts are well developed. The junctional complexes are characterized by the occurrence of desmosomes and fairly shallow occluding junctions indicative of low electrical tightness of the monolayer epithelium (79, 80). When grown on microporous supports, LLC-PK1 epithelia generate a transepithelial electrical resistance of ~150 Ω-cm^2.

During the onset of metabolic acidosis, the elevated renal extraction and catabolism of glutamine is accomplished by a rapid increase in the expression of the cytosolic PEPCK (5, 84) and by more gradual increases in the levels of glutaminase (13, 108) and GDH (107), which occur solely within the cells of the proximal convoluted tubule. The observed time course further supports the critical role of increased gluconeogenesis in the renal synthesis of NH_4^+ and HCO_3^- ions. The more rapid activation of gluconeogenesis may also contribute to the significant decreases in α-ketoglutarate and glutamate concentrations that occur in the renal cortex during the acute onset of acidosis (59). Such changes may contribute to the acute activation of glutamine catabolism by decreasing the product inhibition of the GDH and glutaminase reactions. The development of a cell line that models these metabolic features in vitro was essential to characterize the molecular mechanisms that regulate the expression of the GLS1 and PCK1 genes, which encode the kidney-type glutaminase and the cytosolic PEPCK, respectively, and the associated signal transduction pathways. It also provided a paradigm for understanding how renal proximal tubule cells sense changes in acid-base balance and mediate the cell-specific regulation of gene expression.
receptor subtypes for vasopressin (V₁ and V₂ receptor) (21) and responsiveness to calcitonin and oxytocin (37, 66). Metabolically, LLC-PK₁ cells are unable to perform gluconeogenesis, i.e., to synthesize glucose from lactate or pyruvate, another prominent feature of cells of the proximal tubule. The inability of LLC-PK₁ cells to perform gluconeogenesis is due to a lack of fructose-1,6-bisphosphatase (FBPase), a key enzyme in the gluconeogenic pathway (27). Low levels of the other gluconeogenic enzymes, PEPCK and glucose-6-phosphatase, have been described in LLC-PK₁ cells (27, 63, 66) (Table 1). Moreover, LLC-PK₁ cultures exhibit only a slight ammonia-geneic response to medium that models a metabolic acidosis (25, 69, 82).

Isolation and Biochemical Characterization of LLC-PK₁-FBPase⁺ Cells

In 1985, a gluconeogenic cell line was isolated from the LLC-PK₁ renal epithelial cells using the selective pressure of glucose-free culture conditions (22). LLC-PK₁ cells were initially adapted to a low (< 0.5 mM)-glucose medium over six to eight passages (~10–12 wk). After this adaptation, confluent cultures were switched to essentially glucose-free culture medium (DMEM without glucose, 10% dialyzed fetal bovine serum) supplemented with 10 mM sodium pyruvate. Surviving cells were isolated and propagated in pyruvate-supplemented, glucose-free medium. This adaptation procedure has been reproduced in another laboratory (6).

Furthermore, glucose-free selection was successfully used to isolate a gluconeogenic strain of opossum kidney (OK) cells (29, 40). The OK cell line (ATCC CRL-1840) was initiated in 1975 from a female American opossum (Didelphys virginiana) (51). The cells retain a number of renal proximal tubule functions in culture. In particular, their PTH responsiveness (51). The cells retain a number of enzymatic activities in LLC-PK₁-FBPase⁺ cells described in LLC-PK₁ cells (27, 63, 66) (Table 1).

Metabolic Features of LLC-PK₁-FBPase⁺ Cells

The LLC-PK₁ cells that survived after the glucose-free selection procedure were analyzed for FBPase activity. In contrast to the parental LLC-PK₁ cells, the new strain expressed FBPase activity both under glucose-free culture conditions and when cells were maintained in glucose-containing media (Table 1). Therefore, the isolated gluconeogenic cell strain was designated LLC-PK₁-FBPase⁺ (22). In addition, the PEPCK activity in LLC-PK₁-FBPase⁺ cells is 10-fold higher than in the LLC-PK₁ parental cells (40). PEPCK activity was increased further when the LLC-PK₁-FBPase⁺ cells were cultured with 5 mM glucose (Table 1). The enzymatic activities are in good accord with the activities of the PEPCK promoter in these cells, determined by transient transfection with PEPCK promoter-CAT reporter constructs. The relative promoter activities were 1.4 ± 0.2 in LLC-PK₁ vs. 9.0 ± 2.3 in LLC-PK₁-FBPase⁺ cells (6).

Based upon the enzymatic analyses, it was concluded that the LLC-PK₁-FBPase⁺ cells should be capable of conducting gluconeogenesis. However, net synthesis of glucose from precursor substrates could not be detected. The hexose phosphate intermediates synthesized via gluconeogenesis were probably utilized in the hexose monophosphate shunt for NADPH and ribonucleotide synthesis (23), preventing net release of free glucose. Therefore, metabolic flux through the gluconeogenic pathway was indirectly tested using 3-mercaptopicolinic acid (3-MPA), a specific inhibitor of PEPCK (22). When cells were incubated with substrates preceding the PEPCK step in glucooegenesis (pyruvate, oxaloacetate, α-ketoglutarate), 3-MPA completely inhibited growth and caused lysis and death of cells. However, incubation in the presence of 3-MPA with substrates, which enter the gluconeogenic pathway above PEPCK (dihydroxyacetone, glycerol), had no effect on cell growth. Thus LLC-PK₁-FBPase⁺ cells fully depend on metabolic flow of substrates through gluconeogenesis when incubated in the absence of sugars, and PEPCK is essential when the only substrates present are those that enter the gluconeogenic pathway below the PEPCK reaction.

Further evidence for a functional gluconeogenic pathway in LLC-PK₁-FBPase⁺ cells was obtained in studies that model a metabolic acidosis in vitro (24, 25, 30). When LLC-PK₁-FBPase⁺ cultures were transferred from a normal glucose-free medium (gluconeogenic conditions) to a glucose-free acidic medium (9 mM HCO₃⁻, pH 6.9), the medium pH was not maintained but became more alkaline during a 24-h incubation period. However, when cultures were grown in the presence of 5 mM glucose (glycolytic conditions), the medium pH remained constant at acidic values. The observed alkaline shift in medium pH might be due to enhanced HCO₃⁻ production by the gluconeogenic LLC-PK₁-FBPase⁺ cells in the acidic medium, indicative of metabolic flux from α-ketoglutarate to glucose. In renal proximal tubule cells, gluconeogenesis and ammoniagenesis are tightly coupled, especially during metabolic acidosis.

Comparison of Gluconeogenic LLC-PK₁-FBPase⁺ and OKGNG⁺ Cells

Another metabolic characteristic of LLC-PK₁-FBPase⁺ cells is the fact that they are unable to appreciably utilize lactate, whether produced endogenously or exogenously added as the sodium salt (29, 40). During the isolation procedure, it was recognized that LLC-PK₁-FBPase⁺ cells died when only 5 mM lactate was provided as the primary carbon source under glucose-free culture conditions (22). The differences in lactate and pyruvate utilization could be due to differences in the
subcellular distribution of PEPCK in the two cell lines (29, 40). The PCK1 and PCK2 genes encode the cytoplasmic and mitochondrial isoforms of PEPCK, respectively. The two isoforms participate in separate pathways that differ in the reactions that are used to generate the cytosolic NADH needed to support gluconeogenesis (39). As a result, mitochondrial PEPCK is the preferred isoform to support gluconeogenesis from lactate, while the cytosolic isoform is required to convert pyruvate, glutamine, and TCA cycle intermediates to glucose. Following subcellular fractionation, the majority of PEPCK pyruvate, glutamine, and TCA cycle intermediates to glucose support gluconeogenesis (39). As a result, mitochondrial forms participate in separate pathways that differ in the reac-

mitochondrial isoforms of PEPCK, respectively. The two iso-

LLC-PK1-FBPase 

addition to gluconeogenic competence and pH responsiveness, are characteristic of renal proximal tubular epithelial cells. In gluconeogenic but they also exhibit other unique features that free culture conditions (22), the resulting cells are not only ing only a single selective pressure, namely, growth in glucose-

contrast to the parental LLC-PK1 cells, LLC-PK1-FBPase 

detected in LLC-PK1-FBPase 

diaminoxidase, another proximal tubule-specific enzyme, was 

More recently, enzyme activity and mRNA expression of 

(Feifel E and Gstraunthaler G, unpublished observations).

Although LLC-PK1-FBPase 

cells were isolated by apply-

PK1 epithelia produce an apical positive PDte. This results from 

potential difference (PDte) of about 

1.5 mV, whereas LLC- 

exchanger (1, 87). By 

PK1 wild-type cells, showing slight increases in tetraploidy (21). The pleiotropic phenotype of the gluconeogenic LLC-

PK1-FBPase 

cells is stable, since long-term culture (>50 passages) even in medium containing 5 mM glucose does not revert the cells to a glycolytic metabolism. Thus the cumulative data, which are summarized in Table 2, demonstrate that the altered phenotype of LLC-PK1-FBPase 

cells is pleiotropic.

pH Responsiveness of LLC-PK1-FBPase 

Cells

Besides the “induction” of gluconeogenesis, another prom-

Table 2. Pleiotropic phenotype of LLC-PK1-FBPase 

cells

| Phenotype                                                                 | LLC-PK1-FBPase 
<table>
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<tr>
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<tr>
<td>Growth under glucose-free culture conditions</td>
<td>Enhanced glutamine metabolism</td>
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<tr>
<td>Gluconeogenic competence, metabolic flow through gluconeogenic path</td>
<td>Expression of gluconeogenic key enzymes fructose-1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK)</td>
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<tr>
<td>Increased oxidative metabolism</td>
<td>Increased mitochondrial volume density</td>
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<tr>
<td>Increased phosphate-dependent glutaminase activity</td>
<td>Increased basal ammonia generation</td>
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<tr>
<td>Decreased expression of glycolytic enzymes</td>
<td>pH responsiveness</td>
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<tr>
<td>Adaptive responses to metabolic acidosis in vitro:</td>
<td>Adaptive increase in ammonium ion production</td>
</tr>
<tr>
<td>Adaptive increase in glutaminase enzyme activity and specific glutaminase mRNA levels due to pH-mediated increased mRNA stability</td>
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<tr>
<td>Adaptive increase in PEPCK enzyme activity and mRNA levels of cytosolic PEPPK isoform due to pH-induced enhanced rate of transcription</td>
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<tr>
<td>Apical proton secretion and strong expression of apical membrane Na+/H+ exchanger NHE3 mRNA</td>
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<tr>
<td>Apical negative transcellular potential difference (PDap) and high transferrable cation permeability (PNa &gt;&gt; PCl) coincident with claudin-2 expression</td>
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<tr>
<td>Increased apical membrane surface density</td>
<td>Increased γ-glutamyltransferase activity</td>
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<tr>
<td>Expression of diaminoxidase enzyme activity</td>
<td>Lack of alkaline phosphatase activity</td>
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PK₁-FBPase⁺ cells exhibit an increased mitochondrial volume density (21), which may explain the increased rate of glutamine catabolism that is paralleled by increased basal activity of the mitochondrial phosphate-dependent glutaminase and the greater basal rates of NH₄⁺ ion production (24). Most importantly, when LLC-PK₁-FBPase⁺ cultures were adapted to acidic conditions, the cells respond with a pronounced increase in NH₄⁺ ion production that correlates with a similar increase in assayable glutaminase activity (24). This is in contrast to the parental LLC-PK₁ cells, which exhibit only a slight increase in glutamine metabolism after exposure to acidic medium (25, 30). The LLC-PK₁ cells are primarily glycolytic and catabolize glutamine at much lower rates (28). Furthermore, LLC-PK₁ cells lack any adaptive increase in glutaminase activity. PEPCK enzyme activity was also increased in acid-adapted LLC-PK₁-FBPase⁺ cells (40). Thus the gluconeogenic LLC-PK₁-FBPase⁺ strain is a pH-responsive permanent renal cell line, making it a valuable in vitro model system for studying the molecular mechanisms of renal acid-base adaptation (12, 14) (see below).

LLC-PK₁-FBPase⁺ cells were also used to characterize how the apical catabolism of glutamine and the transport of glutamate affect proximal tubule function (103). Compared with the parental LLC-PK₁ cells, LLC-PK₁-FBPase⁺ cells exhibit an increased expression of γ-glutamyltransferase (γ-GT), which parallels the increase in apical membrane surface area (21, 26). Studies, using cultures grown on plastic dishes or on permeable filter supports (62, 64, 104), established that apical hydrolysis of glutamine by γ-GT and transport of the resulting glutamate form a functional unit that modulates intracellular catabolism of glutamine and paracellular ion permeability (101, 102). The higher γ-GT activity in LLC-PK₁-FBPase⁺ cells resulted in higher intracellular glutamate, which alters glutamine metabolism by inhibiting phosphate-dependent glutaminase and promoting GDH and transaminase reactions (25, 100). However, overall glutamine flux is greater in the LLC-PK₁-FBPase⁺ cells due to the higher glutaminase activity (24).

More recently, troglitazone, a high-affinity ligand for peroxisome proliferator-activated receptor-γ (PPAR-γ), was used to induce intracellular acidification in LLC-PK₁-FBPase⁺ cells (71, 72, 95). PPAR-γ is a nuclear receptor that activates transcription of genes involved in energy homeostasis and differentiation. In LLC-PK₁-FBPase⁺ cells, troglitazone inhibited the NHE, leading to a decrease in intracellular pH, which increased ammoniagenesis (71, 103). The primary effect was shown to be a specific activation of the ERK1/2 signaling pathway by troglitazone, resulting in mitochondrial depolarization, increased generation of acid equivalents, and inhibition of NHE-driven acid extrusion by ERK1/2 (71, 72). In this series of studies, the LLC-PK₁-FBPase⁺ cells served as a valuable model to delineate the signaling cascades associated with PPAR-γ-dependent and -independent effects on renal acid-base metabolism.

**Molecular Biological Properties of LLC-PK₁-FBPase⁺ Cells**

The metabolic properties of LLC-PK₁-FBPase⁺ cells were confirmed at the RNA and protein level. Using probes encoding the rat kidney cytosolic PKC1 and chicken liver mitochondrial PKC2 cDNAs, strong expression of cytosolic PEPCK mRNA was observed in LLC-PK₁-FBPase⁺ cells, while the mitochondrial PEPCK mRNA was barely detectable (40). The unique gluconeogenic nature of the LLC-PK₁-FBPase⁺ cells as assessed by expression of FBPase and cytosolic PEPCK mRNAs is documented in the Northern blot shown in Fig. 2. In a survey of continuous renal cell lines, only LLC-PK₁-FBPase⁺ cells express mRNAs that encode FBPase and the cytosolic isoform of PEPCK. Total RNA isolated from the rat kidney cortex served as a control. Furthermore, when LLC-PK₁-FBPase⁺ cells were incubated in an acidic medium for 18 h, only the cytosolic PEPCK mRNA levels increased, while the mitochondrial PEPCK mRNA levels remained unchanged (24, 40). In subsequent studies, it was shown that the adaptive increase in the cytosolic PEPCK mRNA is mediated by an increased rate of transcription (16, 41, 56), as observed in vivo in the rat kidney (45).

The rat kidney expresses two glutaminase mRNAs, a 5.0-kb and a less abundant 3.4-kb mRNA, both of which encode the KGA variant of glutaminase. The two mRNAs, which are produced by use of alternative polyadenylation signals, are coordinately increased fivefold within 1 day after the onset of acidosis (12, 15, 76, 77). In contrast to PEPCK mRNA, this increase results primarily from an increase in the stability of the KGA mRNA (47, 52–54). Functional studies and RNA gel shift analyses of various deletion constructs were performed using LLC-PK₁-FBPase⁺ cells. This analysis indicated that the pH-responsive stabilization is mediated by two 8-base adenylate-uridylate (AU) sequences in the portion of the 3′-untranslated region that is common to both rat KGA mRNAs. To validate these findings, a tetracycline-responsive promoter system was employed to conduct a pulse-chase analyses of the turnover of a chimeric β-globin-glutaminase (BG-GA) mRNA (85). β-GA mRNA had a half-life of 2.9 h in cells maintained in normal medium, which was increased more than fivefold when the cells were transferred to acidic medium. When the AU elements within the β-GA mRNA were mutated, the rate of degradation in normal medium was not affected, but the mRNA was no longer stabilized following transfer of the cells to acidic medium. By contrast, a construct containing only the AU elements (a 29-bp insert) exhibits both rapid degradation.

**Fig. 2. Expression of fructose-1,6-bisphosphatase (FBPase) and cytosolic PEPCK in various renal cell lines and in the rat kidney.** Cultured cells were incubated in normal (pH 7.4) or acidic medium (pH 6.9) for 18 h. Total RNA samples (20 μg) were electrophoresed, blotted, and hybridized with cDNA probes to rat liver FBPase and rat renal cytosolic PEPCK. FBPase⁺, LLC-PK₁-FBPase⁺ cells; OK, opossum kidney cells; MDCK, Madin-Darby canine kidney cells; LLC-PK₁, LLC-PK₁ pig kidney cells; WKPT, Wistar-Kyoto rat proximal tubular cells; HPT, primary cultures of human proximal tubular cells; CTX, rat kidney cortex; OM, outer medulla; IM, inner medulla.
and pH-responsive stabilization (85). Thus the identified AU sequences contribute to the rapid turnover of KGA mRNA, and are both necessary and sufficient to mediate its pH-responsive stabilization.

By contrast, LLC-PK₁-FBPase⁺ cells primarily express two glutaminase mRNAs that are 5.0 and 4.5 kb in size, which encode the KGA and GAC variants of the GLS1 gene, respectively (76, 77). The two mRNAs are produced by alternative splicing of a common transcript. They share a large stretch of identical coding sequence but encode different C-terminal untranslated regions. The levels of the 4.5-kb GAC mRNA are increased threefold when LLC-PK₁-FBPase⁺ cultures were incubated with acidic medium. This adaptation correlates with the increase in assayable glutaminase activity (see Fig. 5 in Ref. 24). The 5.0-kb KGA mRNA species is constitutively expressed and is not increased in response to treatment with acidic medium (24, 76, 77). The 3ʹ-untranslated region of the 5.0-kb porcine and human KGA mRNAs lacks the eight-base AU sequences that function as the pH-response element in rat KGA mRNA, whereas a highly homologous sequence is present in the 3ʹ-untranslated region of the porcine and human GAC mRNAs (38, 53, 54). The function of the homologous AU sequence was confirmed by determining the half-lives of the two mRNAs in control and acid-adapted LLC-PK₁-FBPase⁺ cells. The apparent half-life of the 4.5-kb GAC mRNA was increased 2.3-fold when LLC-PK₁-FBPase⁺ cells were transferred to acidic medium, while the half-life of the 5.0-kb KGA mRNA was unchanged (24, 76). Thus the pH-responsive stabilization of the 4.5-kb GAC mRNA effectively models the changes in the KGA mRNA that occur in rat renal proximal convoluted tubules (45, 46).

LLC-PK₁-FBPase⁺ cells also recapitulate the pH-induced increase in GDH mRNA levels that occurs in rat kidney during the onset of metabolic acidosis (48, 86). GDH mRNA contains four 8-base AU-sequences in its 3ʹ-untranslated region that are highly homologous to the pH-responsive elements identified in rat KGA mRNA. Rat renal cortex contains a cytosolic protein that binds with high affinity and specificity to the AU sequence (52, 53). In subsequent studies, this protein was identified as ζ-crystallin, a NADPH:quinone reductase (92). Two of the AU sequences in GDH mRNA also bind purified recombinant ζ-crystallin with high affinity. However, overexpression or small interfering (si) RNA knockdown of ζ-crystallin had no effect on the basal half-life or the pH-responsive stabilization of the βG-GA mRNA (47). Thus, despite the fact that ζ-crystallin is the primary protein in extracts of rat kidney cortex (92) and of porcine LLC-PK₁-FBPase⁺ proximal tubule cells that binds to the pH-response element, it appears that ζ-crystallin is not the sole or primary mediator of the rapid degradation or the selective stabilization of the GA and GDH mRNAs. However, both cells express high levels of multiple isoforms of AU1, a destabilizing mRNA binding protein, which also binds to the pH-response element of the glutaminase mRNA with high affinity and specificity (85). Thus the binding of this protein may mediate the rapid turnover and the pH-responsive stabilization of the glutaminase mRNA during metabolic acidosis.

**PH Signaling in LLC-PK₁-FBPase⁺ Cells**

Cytosolic PEPCK is encoded by the single-copy PCK1 gene whose transcription is regulated by the binding of multiple transcription factors to specific sites of the promoter. PEPCK transcription changes very rapidly in a tissue-specific manner in response to activation of various signaling pathways (7). Regulatory elements in the PEPCK promoter may best be understood as “units,” each of which is composed of distinct transcription factor-binding sites in the promoter that function together in response to hormonal or dietary stimuli or changes in acid-base status.

As detailed above, transfer of LLC-PK₁-FBPase⁺ cells to acidic culture media (pH 6.9) caused a rapid induction of the PCK1 gene, resulting in concomitant increases in PEPCK mRNA, protein, and assayable activity (24, 65). Transcription run-off experiments indicated that increased expression of rat renal PEPCK during an acute onset of acidosis is regulated primarily at the level of transcription (45). An increase in PEPCK mRNA is observed in rat kidney within 1 h after the onset of acute acidosis. After 7 h, the increase reaches a maximum of sixfold, which is sustained as the rats become chronically acidic (46). By contrast, confluent and well-differentiated cultures of LLC-PK₁-FBPase⁺ cells exhibit a threefold increase in PEPCK mRNA when transferred to acidic medium for 16 h (41). The initial 490-bp of the PEPCK promoter contains at least 12 different protein-binding sites that mediate its tissue-specific expression during development and its response to various hormones (7, 73). Expression of various PEPCK promoter-chloramphenicol acetyltransferase (PCK-CAT) reporter constructs in LLC-PK₁-FBPase⁺ cells indicated that the CRE-1, P2, and P3(II) elements are required for basal and cAMP-stimulated expression in kidney cells (57, 58). Similar experiments suggested that the pH-responsive stimulation of PEPCK expression is mediated primarily by the CRE-1 and P3(II) elements (41).

A decrease in intracellular pH must initiate a signal that mediates the increase in transcription of the PEPCK mRNA. Specific MAP kinase activators and inhibitors were used to determine the potential role of the ERK1/2, SAPK/JNK, and p38 MAPK pathways in the basal and pH-responsive expression of PEPCK mRNA (16). Anisomycin, a potent activator of p38 MAPK, increased PEPCK mRNA to levels comparable to those observed with acid stimulation. SB203580, a specific p38 MAPK inhibitor, inhibited both the acid- and anisomycin-mediated induction of PEPCK mRNA. In LLC-PK₁-FBPase⁺ cells, only the SB-sensitive p38α isoform is strongly expressed. By contrast, the MEK1/2 inhibitors PD098059 and U0126 did not alter the basal or pH-responsive increase in PEPCK mRNA levels (16). In addition, JNK phosphorylation and JNK activity were decreased when cells were transferred to acidic medium. However, p38 MAPK is phosphorylated and thus activated when LLC-PK₁-FBPase⁺ cells are transferred to acidic medium. One downstream substrate of p38 is ATF-2. ATF-2 is a basic-leucine zipper transcription factor that exhibits increased DNA binding and transcriptional activation following dual phosphorylation by p38 MAPK. ATF-2 was also phosphorylated when LLC-PK₁-FBPase⁺ cells were transferred to acidic medium. This phosphorylation occurred with a slight lag compared with phosphorylation of p38 MAPK and was also blocked by addition of SB203580. The sequence of
the CRE-1 element in the \textit{PKC} promoter (TTACGTCA) is a perfect match to the consensus sequence for an ATF-2 binding site (8). Finally, gel-shift analysis (Fig. 9 in Ref. 16) confirmed that ATF-2 is contained in nuclear extracts of LLC-PK\textsubscript{1}−FBPase\textsuperscript{+} cells that binds to the CRE-1 element.

To further characterize the potential role of the p38 MAPK signaling pathway, a tetracycline-responsive promoter was used to express constitutively active (ca) and dominant negative (dn) forms of MKK3 and MKK6 in transfected LLC-PK\textsubscript{1}−FBPase\textsuperscript{+} cells (70). The two MKKs function upstream of p38 MAPK. Expression of caMKK6 produced an increase in PEPCK mRNA that closely mimicked the effect of treatment with acidic medium and also activated expression of a PEPCK−luciferase reporter construct. Expression of the dnMKKs blocked the phosphorylation of p38 MAPK and the induction of PEPCK mRNA. These experiments firmly established that the pH-responsive increase in PEPCK mRNA transcription is mediated by the p38 MAPK signaling pathway and involves the upstream activation of MKK3 and/or MKK6. Thus the SB-sensitive p38α/ATF-2 signaling pathway is the likely mediator of the pH-responsive induction of PEPCK mRNA transcription in renal LLC-PK\textsubscript{1}−FBPase\textsuperscript{+} cells.

Based upon the existing data, the following model (Fig. 3) was developed to explain the increase in transcription of PEPCK mRNA in the renal proximal convoluted tubule during metabolic acidosis (12, 93). During normal acid-base balance, HNF1 and possibly AP-1 transcription factors are bound to the P2 and P3(II) elements of the PEPCK promoter, respectively. A decrease in intracellular pH leads to activation of the α-isof orm of p38 MAPK, which phosphorylates and activates ATF-2. The activated ATF-2 now binds to the CRE-1 element, where it may recruit additional transcription factors. The resulting complex recruits the appropriate coactivators and polymerase that activate transcription of the \textit{PKC}1 gene (16). This proximal tubule-specific regulatory region has been recently defined as an acidosis regulatory unit (ARU) in the kidney (110).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{acidosis_regulatory_unit}
\caption{Mechanism of pH-responsive activation of PEPCK transcription in LLC-PK\textsubscript{1}−FBPase\textsuperscript{+} cells. A decrease in media pH and HCO\textsubscript{3}\textsuperscript{-} leads to activation of MKK3 and MKK6, which phosphorylate and activate p38 MAPK. The activated p38-MAPK subsequently phosphorylates and activates a transcription factor (ATF-2), which binds to the CRE-1 element within the acidosis-regulatory unit of the PEPCK promoter. Activated transcription also requires the binding of hepatic nuclear factor 1 (HNF-1) to the P2 element and AP-1 to the P3(II) element.}
\end{figure}

\textbf{pH-Responsive Stabilization of PEPCK mRNA}

Transfer of LLC-PK\textsubscript{1}−FBPase\textsuperscript{+} cells to an acidic medium produced an increased expression of PEPCK mRNA that occurred following a pronounced delay and that reached a threefold maximum after 18 h. However, this increase in expression occurred with no evident change in the half-life of the PEPCK mRNA (41). The LLC-PK\textsubscript{1}−FBPase\textsuperscript{+} cells used in previous studies were a mixed population of cells. Thus clonal lines of LLC-PK\textsubscript{1}−FBPase\textsuperscript{+} cells were selected to identify a cell line that exhibits a greater fold-increase in cytosolic PEPCK mRNA and protein (65). When treated with acidic medium, the clonal LLC-PK\textsubscript{1}−FBPase\textsuperscript{+}−9C cells exhibit a more rapid and more pronounced increase in PEPCK mRNA and protein that reached a four- to fivefold increase after 15 and 20 h, respectively. Measurement of the half-lives established that the endogenous PEPCK mRNA turns over rapidly (t\textsubscript{1/2} = 3.2 h) in cells maintained in normal medium but is stabilized twofold when the cells are transferred to acidic medium. The pH-responsive stabilization was reproduced in a Tet-responsive expression of βG-PEPCK-1 mRNA, which contains the entire 3′-untranslated region of PEPCK mRNA. Therefore, the clonal line of LLC-PK\textsubscript{1}−FBPase\textsuperscript{+}−9C cells effectively models both the transcriptional activation and the pH-responsive stabilization of renal PEPCK mRNA. The latter response was lost by mutation of a 17-base AU sequence in the PCK-6 segment of the 3′-untranslated region. This sequence contains a high degree of identity to the two eight-base AU sequences that mediate the pH-responsive stabilization of rat KGA mRNA (53). In addition, 11 of the 17 nucleotides (UUAAAU-UAUUU) are fully conserved within the 3′-end of the 3′-untranslated region of all mammalian \textit{PKC}1 genes that have been sequenced. The PCK-6 segment also binds AUFI and is the primary element that mediates the rapid turnover of PEPCK mRNA (35). Therefore, this highly conserved sequence contributes to the rapid turnover and mediates the pH-responsive stabilization of the PEPCK mRNA.

Electrophoretic mobility shift assays established that purified recombinant HuR, a stabilizing mRNA binding protein, also binds with high affinity and specificity to two sites within the 3′-untranslated region of the PEPCK mRNA (65). These sites overlap with the AUFI binding sites in the PCK-6 and PCK-7 segments (35). siRNA knockdown of HuR in LLC-PK\textsubscript{1}−FBPase\textsuperscript{+}−9C cells caused a pronounced decrease in basal expression and reduced the pH-responsive increases in PEPCK mRNA and protein. Most importantly, the siRNA knockdown of HuR blocked the pH-responsive increase in the half-life of the endogenous PEPCK mRNA. However, treatment with acidic medium had no effect on the level or subcellular distribution of HuR or the various isoforms of AUFI (65). Therefore, the pH-responsive stabilization of PEPCK mRNA may require covalent modifications of HuR and/or AUFI, which affects their binding to the elements that mediate the rapid turnover of PEPCK mRNA. The LLC-PK\textsubscript{1}−FBPase\textsuperscript{+}−9C cells were also used to develop a recruitment assay using chimeric MS2 RNA binding proteins to establish that the concurrent binding of HuR and AUFI is required to mediate the pH-responsive stabilization of PEPCK mRNA (33). Our current understanding of the mechanism by which the PEPCK mRNA is stabilized in response to acidosis is summarized in Fig. 4. The same mechanism is likely to regulate the expression of
Normal Acid-Base Balance

Metabolic Acidosis

Fig. 4. Proposed mechanism for the pH-responsive stabilization of PEPCK mRNA. Interactions between the cap binding proteins (4E and 4G) and the polyA binding protein (PABP) cause mRNA to form a circular structure that enhances translation. Both a stabilizing mRNA binding protein (HuR) and destabilizing mRNA binding protein (AUF1) bind to the adenylate-uridylate (AU)-rich sequences within the 3'-untranslated region (UTR) of the PEPCK mRNA during normal acid-base balance. This complex recruits a deadenylase (Deaden) that shortens the polyA tail and causes dissociation of the polyA binding proteins (PABPs). The deadenylated mRNA is degraded in processing bodies by decapping and degradation from the 5'-end. In response to metabolic acidosis, the extent of phosphorylation of HuR is decreased while AUF1 is phosphorylated at additional sites. These changes lead to increased binding of HuR and a remodeling of the HuR/AUF1 complex that is bound to the 3'-UTR of PEPCK mRNA. The new complex is less effective at recruiting deadenylase and thereby promotes stabilization of the PEPCK mRNA.

multiple proteins in the renal proximal convoluted tubule during the onset of acidosis (78). Given the rapid turnover and demonstrated stabilization, PEPCK mRNA expression in the clonal LLC-PK1-FBPase+9C cells provides an excellent model system and effective paradigm to further characterize the molecular mechanism that mediates a major component of the renal response to acidosis.

Summary and Future Experiments

The renal proximal tubule rivals the liver in its ability to perform gluconeogenesis, i.e., the de novo synthesis of glucose from low-molecular-weight precursors (e.g., pyruvate, lactate, alanine, and glutamine). However, the mechanisms to induce and maintain the key gluconeogenic enzymes in cultured renal proximal tubular cells are largely unknown. Most established renal cell lines (Fig. 1), including RPTEC/TERT1 cells, a nontransformed, telomerase-immortalized human proximal tubular cell line (105), are not gluconeogenic. In addition, the expression of FBPase, PEPCK, and glucose-6-phosphatase is rapidly lost in primary cultures of human (9), rabbit (94), and rat (11) proximal tubular cells, while glycolytic enzyme activities (LDH, hexokinase) are dramatically increased. As a result, gluconeogenesis is not retained even in primary cultures of renal proximal tubules.

Porcine renal epithelial LLC-PK1 cells retain a number of proximal tubule-specific features (31), but they also do not express the key gluconeogenic enzymes and thus are unable to grow in the absence of glucose (27). LLC-PK1-FBPase+ cells were isolated by weaning LLC-PK1 cultures from glucose (22). The selective pressure of culturing cells under essentially glucose-free culture conditions resulted in the induction of FBPase and the increased expression of PEPCK (6). When the LLC-PK1-FBPase+ cells were recultured in the presence of glucose, the gluconeogenic phenotype was retained (Table 1). Thus the LLC-PK1-FBPase+ cells provide a unique model system for studying this important metabolic process and its role in acid-base balance.

The induction of gluconeogenic competence in LLC-PK1-FBPase+ cells may have occurred as an adaptation to the weaning process or, alternatively, the surviving cells were propagated from a preexisting, but minor pool of gluconeogenic cells in a heterogeneous population of LLC-PK1 cells. However, the selection procedure was successfully repeated using a clonal line of LLC-PK1 cells (22) and has been reproduced by others (6). Furthermore, a similar protocol was used to isolate a gluconeogenic line of OK cells (29). Thus it was concluded that LLC-PK1-FBPase+ arose from adaptation. With the present state of knowledge, we can only speculate on the underlying mechanism. As previously noted, gluconeogenesis is highly tissue specific, with maximal activity occurring in hepatocytes, renal proximal tubules, and adipocytes (7, 39). Of the genes encoding the rate-limiting gluconeogenic enzymes, the PCK1 gene has been most intensely studied (55). Expression of cytosolic PEPCK mRNA is primarily regulated by the binding of transcription factors to specific sites in the PCK1 promoter (7, 12, 110). It has been reported that binding of hepatic nuclear factor 1 (HNF-1) to the P2 element of the PCK1 promoter is essential for kidney-specific expression (6, 12, 73).

Glucose depletion (fasting) and glucose-free culture may have activated or increased expression of HNF-1 and/or additional transcription factors that account for the pleotropic phenotype of the LLC-PK1-FBase+ cells. Alternatively, chromatin remodeling or epigenetic modifications of nucleosomal histones may have produced the altered gene expression (3, 17). Changes in the methylation status of histone H3/H4 have been shown to modulate the occupancy of transcription factors on the PCK1 promoter and affect its transcription (110). Whether weaning of LLC-PK1 cells from glucose and subsequent growth in glucose-free culture conditions produced epigenetic effects (36), which resulted in increased expression of gluconeogenic enzymes in LLC-PK1-FBPase+ cells, remains to be determined.

The LLC-PK1-FBPase+ cells have been used to identify some of the RNA binding proteins that mediate the pH-responsive stabilization of glutaminase and PEPCK mRNAs (47). Recent experiments indicate that increased stability results from covalent modification and remodeling of the protein complex that associates with the functional AU element in the mRNAs (33). Future experiments using a photostable crosslinker and RNA pull-downs may determine how changes in acid-base balance affect the composition of this complex and the mode and sites of covalent modifications that regulate its
function. Microarray analysis of the immunoprecipitated RNAs would identify additional mRNAs that bind this complex and contribute to the renal response to acidosis or alkalosis. Recent proteomic studies have identified nearly 100 proteins that are significantly increased in the rat renal proximal tubule during acute and chronic acidosis (18, 83, 97). A similar analysis of the response of the transcriptome and proteome of LLC-PK1-FBPase* cells would greatly expand our knowledge of how effectively this cell line models the characteristics and the responses of the renal proximal tubule.

A detailed metabolomic analysis of these cells may also identify the mechanisms that mediate the rapid activation of the mitochondrial glutaminase and glutamine metabolism that occur during an acute onset of acidosis and that precede the more well-characterized increases in gene expression. Thus there are numerous ways in which this unique cell line can be studied to well-characterized increases in gene expression. Thus there are mitochondriaally glutaminase and glutamine metabolism that occur during an acute onset of acidosis and that precede the more well-characterized increases in gene expression. Thus there are numerous ways in which this unique cell line can be studied to increase our understanding of the metabolism and function of the renal proximal tubule.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.P.C. and G.G. provided conception and design of research; N.P.C. and G.G. performed experiments; N.P.C. and G.G. analyzed data; N.P.C. and G.G. drafted manuscript; N.P.C. and G.G. edited manuscript; N.P.C. and G.G. interpreted results of experiments; N.P.C. and G.G. helped to establish LLC-PK1-FBPase* cell line in his laboratory. Thanks are also expressed to all of the researchers participating in the projects.

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