Mechanism of increased renal gene expression during metabolic acidosis

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Curthoys, Norman P., and Gerhard Gstraunthaler. Mechanism of increased renal gene expression during metabolic acidosis. Am J Physiol Renal Physiol 281: F381–F390, 2001.—Increased renal catabolism of plasma glutamine during metabolic acidosis generates two ammonium ions that are predominantly excreted in the urine. They function as expendable cations that facilitate the excretion of acids. Further catabolism of α-ketoglutarate yields two bicarbonate ions that are transported into the venous blood to partially compensate for the acidosis. In rat kidney, this adaptation is sustained, in part, by the induction of multiple enzymes and various transport systems. The pH-responsive increases in glutaminase (GA) and phospho(enol)pyruvate carboxykinase (PEPCK) mRNAs are reproduced in LLC-PK1-fructose 1,6-bisphosphatase (FBPase) cells. The increase in GA activity results from stabilization of the GA mRNA. The 3'-untranslated region of the GA mRNA contains a direct repeat of an eight-base AU sequence that functions as a pH-response element. This sequence binds ζ-crystallin/NADPH:quinone reductase with high affinity and specificity. Increased binding of this protein during acidosis may initiate the pH-responsive stabilization of the GA mRNA. In contrast, induction of PEPCK occurs at the transcriptional level. In LLC-PK1-FBPase + kidney cells, a decrease in intracellular pH leads to activation of the p38 stress-activated protein kinase and subsequent phosphorylation of transcription factor ATF-2. This transcription factor binds to CAMP-response element 1 within the PEPCK promoter and may enhance its transcription during metabolic acidosis.

glutamine metabolism; glutaminase; phospho(enol)pyruvate carboxykinase; LLC-PK1-fructose 1,6-bisphosphatase cells

RENAL GLUTAMINE METABOLISM

GLUTAMINE IS AN IMPORTANT metabolic fuel that is constitutively metabolized in liver (34), intestinal epithelium (55), lymphocytes (59), brain (48), and various transformed cells (9). In contrast, renal catabolism of glutamine is activated only during metabolic acidosis (77). During normal acid-base balance, the kidney extracts and metabolizes very little of plasma glutamine (75). The measured renal arteriovenous difference for plasma glutamine is normally <3% of arterial concentration. However, ~20% of plasma glutamine is filtered by the glomeruli and enters the lumen of the nephron. The filtered glutamine is reabsorbed primarily by the epithelial cells of the proximal convoluted tubule (72). It is initially transported across the apical brush-border membrane, and subsequently most of the recovered glutamine is returned to the blood via transport across the basolateral membrane. The specific transporters that are responsible for the transcellular flux of glutamine have not been identified.

Utilization of the small fraction of extracted plasma glutamine requires its transport into the mitochondrial matrix, where glutamine is deamidated by glutamin-
ase (GA) and then oxidatively deaminated by glutamate dehydrogenase (GDH). Glutamine uptake occurs via a mersalyl-sensitive electroneutral uniporter (68). The mitochondrial glutamine transporter was recently purified from rat kidney and shown by reconstitution in lipid vesicles to be specific for glutamine and asparagine and inhibited by various thiol reagents (43). Kinetic measurements indicated that the rate of glutamine transport in isolated rat renal mitochondria is not rate limiting for glutamine catabolism (19, 47).

However, the activity of either the mitochondrial glutamine transporter or GA must be largely inactivated in vivo during normal acid-base balance to account for the effective reabsorption of glutamine. During normal acid-base balance, approximately two-thirds of the ammonium ions produced from glutamine are trapped in the tubular lumen and are excreted in a slightly acidified urine (74).

**ACUTE ACIDOSIS**

The maintenance of blood acid-base balance is essential for survival. Increased renal ammoniagenesis and gluconeogenesis from plasma glutamine constitute an adaptive response that partially restores acid-base balance during metabolic acidosis (3). Thus the renal catabolism of glutamine is rapidly activated after the acute onset of metabolic acidosis. Within 1–3 h, the arterial plasma glutamine concentration is increased twofold (39), due primarily to an increased release of glutamine from muscle tissue (70). Significant renal extraction of glutamine becomes evident as the arterial plasma concentration is increased. Net extraction reaches 30% of the plasma glutamine, a level that exceeds the percentage filtered by the glomeruli. Thus the direction of the basolateral glutamine transport must be reversed in order for the proximal convoluted tubule cells to extract glutamine from both the glomerular filtrate and the venous blood. In addition, the transport of glutamine into the mitochondria may be acutely activated (69). Further responses include a prompt acidification of the urine that results from an acute activation of the apical Na⁺/H⁺ exchanger (38). This process facilitates the rapid removal of cellular ammonium ions (78) and ensures that the bulk of the ammonium ions generated from the amide and amine nitrogen of glutamine are excreted in the urine. Finally, a pH-induced activation of α-ketoglutarate dehydrogenase reduces the intracellular concentrations of α-ketoglutarate and glutamate (54). Thus increased catabolism initially results from a rapid activation of key transport processes, an increased availability of glutamine, and a decreased concentration of the products of the GA and GDH reactions.

**CHRONIC ACIDOSIS**

During chronic metabolic acidosis, many of the acute adaptations are partially compensated for and the arterial plasma glutamine concentration is decreased to 70% of normal (3). However, the kidney continues to extract more than one-third of the total plasma glutamine (75) in a single pass through the organ (Fig. 1). Renal catabolism of glutamine is now sustained by increased expression of the genes that encode mitochondrial GA (10), mitochondrial GDH (83), and cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK) (4). The tubular distributions of PEPCK, fructose 1,6-bisphosphatase (FBPase), and glucose 6-phosphatase were determined in microdissected segments of the rat kidney.

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**Fig. 1. Pathway of renal glutamine catabolism during chronic acidosis.** Increased renal catabolism of glutamine is sustained during chronic acidosis by increased expression of the genes that encode the glutaminase (GA), the glutamate dehydrogenase (GDH), the phosphoenolpyruvate carboxykinase (PEPCK), and the mitochondrial glutamine transporter, the apical Na⁺/H⁺ exchanger, and the basolateral Na⁺-3HCO₃⁻ cotransporter. Arrows, increased activities; AV, arteriovenous difference; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate.
nephron (30). All three activities, and thus the pathway of gluconeogenesis, are expressed solely within the proximal tubule. However, after the onset of acidosis, the rapid increase in PEPCK occurs only within the S1 and S2 segments of the proximal tubule (4). The more gradual increase in the levels of the mitochondrial GA (10, 84) and GDH (83) also occurs solely within the proximal convoluted tubule. Previous micropuncture studies (67) and assays using microdissected nephron segments (22) have also established that the preponderance of renal ammoniagenesis in normal or acidic rats occurs within the convoluted portion of the proximal tubule. The decreases in plasma pH and HCO₃⁻ concentration during metabolic acidosis produce a comparable and sustained decrease in the intracellular pH (pHᵰ) within the proximal convoluted tubule (1, 66). Thus the adaptive increases in GA and PEPCK activities may be initiated by a decrease in pHᵰ. Both adaptations result from increased rates of synthesis of the proteins (45, 80) that correlate with comparable increases in the levels of their respective mRNAs (7, 41). However, the increase in GA results from the selective stabilization of the GA mRNA (31, 49, 51), whereas the increase in PEPCK activity results from enhanced transcription of the PEPCK gene (33). The levels of the mitochondrial glutamine transporter (69), the apical Na⁺/H⁺ exchanger NHE3 (38), the basolateral Na⁺-3HCO₃⁻ cotransporter (62), and the apical Na⁺-dicarboxylate cotransporter NaDC-1 (2) are also increased during chronic acidosis. The adaptation in the latter transporter results in increased reabsorption and metabolism of citrate within the proximal tubule. After cellular uptake, citrate is metabolized through one of two pathways, a cytoplasmic pathway involving citrate lyase or a mitochondrial pathway involving the citric acid cycle (73). The onset of acidosis causes an increase in the activities of cytoplasmic citrate lyase and mitochondrial aconitase (2). Both of these pathways generate metabolites that lead to enhanced gluconeogenesis. The increase in apical Na⁺/H⁺ exchanger activity sustains the acidification of the fluid in the tubular lumen and ensures the urinary excretion of the ammonium ions. Thus the increased renal ammoniagenesis continues to provide an expendable cation that facilitates the excreting of titratable acids while conserving sodium and potassium ions. The increased Na⁺/H⁺ exchanger activity also promotes the tubular reabsorption of HCO₃⁻ ions. In addition, the α-ketoglutarate generated from glutamine is converted to glucose. This process generates 2 HCO₃⁻ ions/mol α-ketoglutarate. The increase in basolateral Na⁺-3HCO₃⁻ cotransporter activity facilitates the translocation of reabsorbed and de novo synthesized HCO₃⁻ ions into the renal venous blood. Thus the combined adaptations also create a net renal release of HCO₃⁻ ions that partially compensates for the systemic acidosis. Finally, a recent analysis using cDNA microarrays indicated that renal expression of a large number of genes is altered during metabolic acidosis (82). Of particular interest was the observation that mRNAs encoding proteins that participate in signal transduction and growth regulation were among those that exhibited the greatest response. Such adaptations may contribute to the renal hypertrophy that also occurs during chronic acidosis (61).

Thus the characterization of the molecular mechanisms that regulate expression of the GA and PEPCK genes and the associated signal transduction pathway should provide a paradigm for understanding how the renal proximal convoluted tubule senses changes in acid-base balance and mediates the cell-specific regulation of gene expression.

LLC-PK₁-FBPase⁺ CELLS

Molecular analyses of the mechanisms that mediate the enhanced expression of the GA and PEPCK genes during metabolic acidosis required the identification of a renal cell line that retained the pH-responsive adaptations. LLC-PK₁ cells exhibit a number of properties that are characteristic of renal proximal tubular cells (23), including a pH-responsive increase in glutamine metabolism (24, 28). However, LLC-PK₁ cells are unable to synthesize glucose from lactate or pyruvate due to a lack of FBPase (29). A gluconeogenic subline was isolated by initially adapting LLC-PK₁ cells to low-glucose medium (<0.5 mM) and then selecting them with essentially glucose-free medium supplemented with 10 mM sodium pyruvate. The cells that survived and replicated in the glucose-free selection medium expressed significant FBPase activity when subsequently grown in either the absence or presence of 5 mM glucose. Therefore, the isolated gluconeogenic strain was designated as LLC-PK₁-FBPase⁺ cells (26). The selected cells also exhibited a 10-fold higher level of PEPCK activity than the parental LLC-PK₁ cells (36). Net glucose synthesis from precursor substrates could not be detected. However, treatment of LLC-PK₁-FBPase⁺ cells growing on pyruvate, oxaloacetate, or α-ketoglutarate with 3-mercaptopicolinic acid, a specific inhibitor of PEPCK (26), caused cell lysis and death. However, 3-mercaptopicolinic acid had no effect on cells grown on dihydroxyacetone or glycerol, substrates that enter the gluconeogenic pathway after PEPCK. Thus LLC-PK₁-FBPase⁺ cells are capable of sufficient gluconeogenesis to grow and replicate in the absence of sugars, and PEPCK activity is essential when grown on metabolites that enter the gluconeogenic pathway before the PEPCK reaction.

The parental LLC-PK₁ cells exhibit a slow rate of glutamine catabolism that is only increased slightly when they are grown in acidic medium (24, 28). This increase occurs without an adaptive increase in mitochondrial GA activity (28). In contrast, LLC-PK₁-FBPase⁺ cells exhibit an enhanced oxidative metabolism, an increased mitochondrial density (25), and a parallel increase in basal GA activity. As a result, the LLC-PK₁-FBPase⁺ cells exhibit an enhanced rate of glutamine catabolism (26) and a higher basal rate of ammonia production (27). Most importantly, when transferred to acidic medium (pH 6.9, 9 mM HCO₃⁻), LLC-PK₁-FBPase⁺ cells responded with a pronounced increase in ammonium ion production that correlated
with a similar increase in GA activity (27). PEPCK activity was also increased in acid-adapted LLC-PK\(_1\)-FBPase\(^+\) cells (36). Thus the gluconeogenic LLC-PK\(_1\)-FBPase\(^+\) strain is a pH-responsive renal proximal tubulelike cell line.

The primary difference between the cell culture system and the well-characterized pathway of rat renal glutamine catabolism is the fate of the amine nitrogen. In rat kidney, the glutamate generated by mitochondrial GA during acidosis is deaminated by GDH, thereby generating two ammonium ions per glutamine. In contrast, in both the LLC-PK\(_1\) (28) and LLC-PK\(_1\)-FBPase\(^+\) (58) cells, the resulting glutamate is primarily transaminated to pyruvate to form alanine and \(\alpha\)-ketoglutarate. This pathway yields only one ammonium ion per glutamate.

LLC-PK\(_1\)-FBPase\(^+\) cells express both the cytosolic and the mitochondrial forms of PEPCK mRNAs (36). However, when cells were incubated in acidic media for 18 h, the level of the cytosolic PEPCK mRNA was increased threefold whereas the level of the mitochondrial PEPCK mRNA was unchanged. Rat kidney contains two GA mRNAs, a 4.7-kb and a less abundant 3.4-kb mRNA, that are coordinately affected in response to changes in acid-base balance (41, 42). In LLC-PK\(_1\)-FBPase\(^+\) cells, the primary GA mRNAs are 5.0 and 4.5 kb in length (60). However, only the 4.5-kb GA mRNA is increased (3-fold) when LLC-PK\(_1\)-FBPase\(^+\) cultures were incubated with acidic culture media. This increase correlates with the increase in assayable GA activity. The 5.0-kb GA mRNA is constitutively expressed and remains unaltered (27, 60). The two GA mRNAs are produced by alternative splicing of a single transcript (Taylor L and Curthoys NP, unpublished observations). The resulting mRNAs contain distinct 3'-nontranslated regions and encode GA isoforms that have different COOH-terminal sequences. The 3'-nontranslated region of the 5.0-kb porcine GA mRNA lacks the eight-base AU sequence that mediates the pH-responsive stabilization of the rat GA mRNA (60). However, the 4.5-kb GA mRNA, which is the homolog of the human GAc mRNA (14), contains two separate pH-response elements. This finding is consistent with the observation that the apparent half-life of the 4.5-kb GA mRNA was increased 2.3-fold when LLC-PK\(_1\)-FBPase\(^+\) cells were transferred to acidic media (27, 60). Thus the LLC-PK\(_1\)-FBPase\(^+\) cells effectively model the adaptive changes in both PEPCK and GA mRNAs that are observed in rat renal proximal tubular cells. A pH-induced increase in the levels of GDH mRNA also occurs in LLC-PK\(_1\)-FBPase\(^+\) cells (46).

The LLC-PK\(_1\)-FBPase\(^+\) cells exhibit a pleiotropic phenotype compared with the parental LLC-PK\(_1\) cells. In addition to being gluconeogenic and pH responsive, the LLC-PK\(_1\)-FBPase\(^+\) cells also exhibit a greater apical proton secretion (27). This correlates with increased levels of the NHE3 mRNA that encodes the apical Na\(^+\)/H\(^+\) exchanger (Feifel E and Gstraunthaler G, unpublished observations). LLC-PK\(_1\)-FBPase\(^+\) cells cultured on permeable supports generate an apical negative transepithelial potential difference of \(-1.5\) mV, whereas LLC-PK\(_1\) epithelia produce an apical positive transepithelial potential difference. The observed difference results from different transepithelial ion permeabilities. LLC-PK\(_1\)-FBPase\(^+\) epithelia are cation selective, whereas parental LLC-PK\(_1\) monolayers are primarily anion selective (40). More recently, another proximal tubule-specific enzyme, diaminooxidase, and its mRNA were detected uniquely in LLC-PK\(_1\)-FBPase\(^+\) cells (Wilflingseder D, Gstraunthaler G, and Schwelberger H, unpublished observations). However, LLC-PK\(_1\)-FBPase\(^+\) cells do not express alkaline phosphatase activity (25).

**STABILIZATION OF GA mRNA**

In the rat, the onset of acidosis leads to a gradual and cell-specific increase in the activity of mitochondrial GA. The 7- to 20-fold adaptation within the proximal convoluted tubule requires 5–7 days (11). Previous studies indicate that the increase in GA protein is due to an increase in its relative rate of synthesis (80), which correlates with an increase in the level of translatable (81) and total (41) GA mRNA. The proximal promoter region of the rat renal GA gene lacks an identifiable TATA box (79). Computer analysis of the initial 2.3-kb segment of the promoter identified a number of putative binding sites for known transcription factors that may contribute to basal and activated transcription. However, nuclear run-off assays indicated that the rate of transcription of the renal GA gene is unaffected by alterations in acid-base balance (41, 42). These observations suggest that the increase in GA activity during chronic acidosis may result from an increased stability of the GA mRNA. The 3.4- and 4.7-kb GA mRNAs found in rat kidney are produced by the use of alternative polyadenylation sites. The levels of the two mRNAs are coordinately affected in response to changes in acid-base balance (41, 42).

The selective stabilization of the GA mRNA was initially demonstrated by stable transfection of various \(\beta\)-globin (\(\beta\)G) constructs into LLC-PK\(_1\)-FBPase\(^+\) cells (31). Expression of \(\beta\)G produced a high level of a very stable mRNA \((t_{1/2} > 30\) h) that was not affected by transfer of the cells to acidic medium. In contrast, \(\beta\)G-GA, which includes the 956-base segment of 3'-nontranslated sequence that is contained in both GA mRNAs, was expressed at significantly lower levels. The decreased expression resulted from the more rapid turnover \((t_{1/2} = 4.6\) h) of the \(\beta\)G-GA mRNA. Transfer of the latter cells to acidic medium resulted in a pronounced stabilization (6-fold) and a gradual induction of the \(\beta\)G-GA mRNA. These studies indicated that the 3'-nontranslated segment contains a pH-response element (pH-RE).

Experiments using additional chimeric \(\beta\)G constructs indicated that a 340-base segment of the GA mRNA, termed R-2, retained most of the functional characteristics of the 3'-nontranslated region (51). Mapping studies, using RNA gel-shift assays, demonstrated that the specific binding site within the R-2
RNA consisted of a direct repeat of an eight-base AU sequence. Site-directed mutation of the direct repeat of the eight-base AU sequence completely abolished the pH-responsive stabilization of the βG-GA mRNA (49). A βG reporter construct that contained the 3’-nontranslated region of the PEPCK mRNA, βG-PCK, was designed to further test the function of the AU element. When expressed in LLC-PK₁-FBPase⁺ cells, the half-life of the βG-PCK mRNA was only slightly stabilized (1.3-fold) by growth in acidic medium. However, insertion of short segments of GA cDNA containing either the direct repeat or a single eight-base AU-sequence was sufficient to impart a fivefold pH-responsive stabilization to the chimeric mRNA. Thus either the direct repeat or a single copy of the eight-base AU sequence is both necessary and sufficient to function as a pH-RE.

The apparent binding to the pH-RE is increased threefold in cytosolic extracts prepared from LLC-PK₁-FBPase⁺ cells that were grown in acidic medium (50). Extracts prepared from the renal cortex of rats that were made acutely acidic also exhibit a similar increase in binding to the direct repeat of the pH-RE. The time course for the increase in binding correlates with the temporal increase in GA mRNA. Scatchard analysis indicated that the increased binding is due to an increase in both the affinity and the maximal binding of the pH-RE binding protein.

A biotinylated oligoribonucleotide containing the pH-RE was used as an affinity ligand to purify a 36-kDa protein from rat renal cortex (76). The isolated protein retained the same specific binding properties as observed with crude cytosolic extracts. Microsequencing of the purified protein by mass spectroscopy identified eight peptides that are contained in mouse ζ-crystallin/NADPH:quinone reductase. In addition, three peptides that differed by a single amino acid from sequences found in mouse ζ-crystallin/NADPH:quinone reductase were identified. The observed differences may represent substitutions found in the rat homolog. Specific antibodies to ζ-crystallin/NADPH:quinone reductase blocked formation of the complex produced by the pH-RE and the purified protein. The 3’-nontranslated region of the GDH mRNA contains four 8-base AU-rich segments in which 7 of the 8-bases are identical to the pH-RE sequence identified in the GA mRNA. The purified ζ-crystallin/NADPH:quinone reductase binds to two of the sequences with high affinity and specificity (Schroeder J, Liu W, and Curthoys NP, unpublished observations). Furthermore, a chimeric βG-GDH mRNA exhibits a pH-responsive stabilization when stably expressed in LLC-PK₁-FBPase⁺ cells. Thus increased binding of ζ-crystallin/NADPH:quinone reductase to the GA and GDH mRNAs may initiate their stabilization and increased expression during acidosis.

ζ-Crystallin/NADPH:quinone reductase constitutes 10% of the total protein present in the lens of hystri- comorph rodents (64) and camelids (18). In these species, the ζ-crystallin gene contains an alternative promoter that accounts for its lens-specific overexpression (21, 52). Similar to other lens crystallins with a limited phylogenetic distribution, ζ-crystallin also has a catalytic activity and is expressed at enzymatic levels in various tissues of different species (20, 65). ζ-Crystallin possesses a novel NADPH-dependent quinone oxidoreductase activity that reduces various quinones through the sequential transfer of single electrons (64).

ζ-Crystallin/NADPH:quinone reductase was not previously known to function as an RNA binding protein. However, bovine ζ-crystallin also binds with high affinity to Z-DNA and to single-strand DNA (17, 63). This interaction is effectively competed by NADPH, suggesting that the DNA may interact with the dinucleotide binding site. Similar interactions have been reported for other enzymes that utilize pyridine nucleotides as a substrate, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase, GDH, thymidylate synthetase, dihydrofolate reductase, and catalase (35, 63). GAPDH has also been reported to bind to AUUU-rich sequences within the 3’-nontranslated regions of mRNAs. Thus the nucleotide binding site of metabolic enzymes may function both in catalysis and in gene regulation (35).

The cumulative data are consistent with the following model (Fig. 2). In normal acid-base balance, the pH-RE present in the 3’-nontranslated region of the GA or GDH mRNA recruits a site-specific endoribonuclease. The onset of metabolic acidosis causes an increase in the binding affinity of ζ-crystallin/NADPH:quinone reductase for the pH-RE. This, in turn, confers increased protection to the GA mRNA from endonucleolytic cleavage and results in an increased stabilization of the GA mRNA. This hypothesis represents the simplest interpretation of the available data. However, the present data do not rule out more complex mechanisms. Further characterization of the actual mechanism will be facilitated through the development of in vitro mRNA decay assays and the use of recombinant ζ-crystallin/NADPH:quinone reductase.

ACTIVATION OF PEPCK TRANSCRIPTION

The increase in the level of PEPCK mRNA in rat kidney is initiated within 1 h after onset of acute acidosis and reaches a maximum within 7 h at a level that is sixfold greater than normal (41). The sixfold induced level of PEPCK mRNA is sustained in rats that are made chronically acidic for periods up to 7 days (42). The observed changes in PEPCK mRNA levels are closely correlated with earlier data that measured changes in the relative rates of PEPCK protein synthesis in normal and acidic rats (45). Thus regulation of the translation of the PEPCK mRNA is unlikely to contribute to the observed changes in PEPCK gene expression. Furthermore, the apparent t₁/₂ for the renal PEPCK protein (~6 h) is not altered during acidosis. However, transcription run-off experiments using isolated rat renal nuclei indicated that transcription of the PEPCK gene was rapidly activated after the onset of acute acidosis (41). The initial increase in the relative rate of transcription both preceded and exceeded the initial increase in PEPCK
mRNA. Therefore, activated transcription can fully account for the initial induction of PEPCK mRNA.

The cytosolic form of PEPCK is expressed predominantly in liver, kidney, and adipose tissues (32). Transcription of PEPCK is suppressed during fetal development but is dramatically induced in liver at birth. Transcription of the PEPCK gene in the postnatal liver is stimulated by glucagon via cAMP, thyroid hormone, retinoic acid, and glucocorticoids, whereas insulin and phorbol esters inhibit its expression. Expression of a chimeric PEPCK-bovine growth hormone gene in transgenic mice revealed that a relatively small region within the PEPCK promoter (−460 to +73 bp) contains most of the information required for conferring the appropriate pattern of developmental, hormonal, and dietary regulation of the PEPCK gene in liver (56, 57). A larger 2-kb segment of the PEPCK promoter was required to drive expression of the transgene in adipose tissues (57, 71). However, both of these constructs were expressed at relatively low levels in the kidney.

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 (37). Similar cultures transfected with PEPCK−490 chloramphenicol acetyltransferase (CAT) exhibit a two- to threefold increase in CAT activity when shifted to acidic medium for 48 h. Mutation of the P2 promoter element that binds HNF-1 caused an eightfold decrease in basal CAT activity but did not affect the pH response. In contrast, mutation of the P3(II) element that contains an AP-1 site or the CRE-1 site had little effect on basal activity but did not affect the pH response. Deletions or mutations of the other well-characterized elements of the PEPCK promoter had no effect on either activity. These results suggested that changes in the activity or levels of proteins in renal proximal tubular epithelial cells that bind to the P3(II) and CRE-1 elements may mediate the increased transcription of the PEPCK gene during metabolic acidosis. Cassuto et al. (5) used a PCK−597CAT construct along with various deletions and site-specific mutations to reexamine this question. They again found that the wild-type construct exhibited a 2.5-fold increase in CAT activity when the cells were transferred to acidic medium. However, this response was retained in constructs that lacked the entire P3 and P4 region or contained a mutation in the P1 element. A mutation in CRE-1 again caused a partial reduction in the fold-response but this was due largely to an increase in basal activity. They again observed that mutation of the P2 element significantly reduced basal activity. Because they also observed that the P2 mutant had a lower pH response (1.6-fold), they concluded that the binding of HNF-1 to this element may contribute to both basal and pH-responsive activation of PEPCK expression in kidney cells. Finally, Drenowska et al. (12) compared the pH responsiveness of PCK−490CAT and PCK−2300CAT constructs in subconfluent LLC-PK1-FBPase+ cells. They reported that a significant pH-response was observed only with the construct containing the longer promoter element.

Given the divergence of these results and the marginal sensitivity of the CAT-reporter constructs, the various promoter segments were cloned into pGL2-Basic (Promega), which encodes the firefly luciferase gene (Wall Q-T and Curthoys NP, unpublished observations). The activity of the resulting PCK−2300Luc construct in LLC-PK1-FBPase+ cells is activated 30-fold by coexpression of the catalytic subunit of PKA (53). This response maps primarily to the CRE-1 element and is mediated, in part, by C/EBPb. In contrast, neither PCK−490Luc nor PCK−2300Luc exhibited a pH-responsive activation. Stable cell lines that express integrated copies of the PEPCK promoter/luciferase gene were also isolated and tested for a pH response. Again, none of these constructs exhibited a pH-responsive increase in luciferase activity even though the level of the endogenous PEPCK mRNA was still increased threefold after transfer to acidic medium. Similar experiments were performed using adenovirus constructs to infect LLC-PK1-FBPase+ cells with the PCK−2300Luc gene. Control experiments using a green fluorescent protein construct indicated that nearly all
the LLC-PK1-FBPase<sup>+</sup> cells are rapidly infected with the adenovirus. This protocol also produced very high luciferase activity in the LLC-PK1-FBPase<sup>+</sup> cells, but again this activity was not pH responsive. Therefore, elements outside the proximal promoter may also be involved in this response.

A PEPCK transgene developed by Eisenberger et al. (13) exhibits a normal developmental profile and appropriate adult levels of expression in kidney. This construct contains the entire rat PEPCK gene including 362 bp of the 5′-promoter region and 0.5 kb of the 3′-flanking sequence. It differed from the normal rat gene only by the removal of a 465-bp EcoRI/SphI fragment from the exon encoding the 3′-nontranslated region and insertion of the corresponding segment from the chicken PEPCK gene. In contrast, transgenes that used longer promoter segments to drive expression of a reporter gene were weakly expressed in kidney (56, 57, 71). Furthermore, the level of the hybrid rat and chicken PEPCK mRNA was increased in the kidneys of the transgenic mice that were made acidic (Reshef L, personal communication). These observations strongly suggest that an element downstream of the basal promoter is essential for normal expression of the PEPCK gene in kidney cortex. More recently, Cissel and Chalkley (8) mapped the nuclease-hypersensitive sites within the chromatin structure of the PEPCK gene in rat kidney and in NRK52E cells, a rat kidney cell line that does not express the PEPCK gene. The −6,200−, −4,800−, −1,240−, and +4,650-bp hypersensitive sites previously identified in rat liver DNA (44) were not present in kidney DNA. In contrast, DNA from normal and acidic rats exhibited hypersensitive sites at −3,100, −400/+1, +1,900, and +6,200 bp. However, the −3,100− and +6,200-bp sites were also present in DNA isolated from the NRK52E cells. Thus only the proximal promoter (−400/+1) region and the unique site at +1,900 bp correlated with kidney-specific expression of the PEPCK gene. The latter site is contained within intron 4 of the rat PEPCK gene. This site may contain the additional element that is needed for enhanced expression and pH-responsive induction in kidney cells.

**SIGNAL TRANSDUCTION**

A decrease in pH<sub>i</sub> must initiate a signal that mediates the increase in transcription of the PEPCK mRNA. The potential involvement of known mitogen-activated protein kinase (MAPK) activities [extracellular signal-regulated kinase (ERK)1/2, c-Jun NH<sub>2</sub>-terminal kinase (JNK), p38 stress-activated protein kinase (SAPK)] was examined by determining the effects of specific MAPK activators and inhibitors on basal and acid-induced PEPCK mRNA levels (Feifel E, Euler S, Obexer P, Tang A, Wei Y, Schramek H, Curthoys NP, and Gstraunthaler G, unpublished observations). The protein synthesis inhibitor, anisomycin, is a potent activator of the p38 SAPK. Anisomycin increased PEPCK mRNA to levels comparable to those observed with acid stimulation. Transfer of LLC-PK1-FBPase<sup>+</sup> cultures to acidic medium resulted in phosphorylation, and thus activation, of p38 SAPK. ATF-2 is a basic leucine zipper transcription factor that activates transcription after dual phosphorylation by p38 SAPK. Phosphorylation of ATF-2 was also observed in LLC-PK1-FBPase<sup>+</sup> cells after treatment with acidic medium. SB-203580, a specific p38 SAPK inhibitor, produced a dose-dependent inhibition of both the acid- and the anisomycin-mediated induction of PEPCK mRNA and blocked phosphorylation of ATF-2. In contrast, the ERK1/2 inhibitors PD-098059 and U-0126 did not affect basal or acid-induced PEPCK mRNA levels. Similarly, the JNK-specific inhibitor curcumin had no effect. In addition, JNK phosphorylation and JNK activity were decreased in acid-adapted cells, indicating that neither ERK1/2 nor JNK plays a significant role. Of the four known p38 SAPKs, only the SB-sensitive α-isof orm is strongly expressed in LLC-PK1-FBPase<sup>+</sup> cells. The octanucleotide sequence of the CRE-1 site of the PEPCK promoter (TTACGTCA) is a perfect match for the consensus element for binding
ATF-2 (6). Gel-shift analysis using a labeled oligonucleotide containing the CRE-1 element produced a band that was partially supershifted with antibodies specific for ATF-2. Thus the SB-sensitive p38 SAPK/ATF-2 signaling pathway may contribute to the pH-responsive induction of PEPCK mRNA transcription in renal LLC-PK1-FBPase⁺ cells.

On the basis of existing data, the following model (Fig. 3) was developed as a hypothesis for the mechanism by which PEPCK mRNA transcription is induced in the renal proximal convoluted tubule during metabolic acidosis. During normal acid-base balance, only the P2 and possibly the P3(II) regions of the PEPCK promoter are occupied with bound transcription factors (5). A decrease in pH leads to activation of the α-isomers of p38 SAPK that, in turn, phosphorylates and activates ATF-2. The activated ATF-2 binds to the CRE-1 element and recruits or interacts with an additional transcription factor that binds downstream of the promoter. The resulting complex recruits the appropriate coactivator and polymerase that are necessary for transcriptional activation of the PEPCK gene.

The pH-responsive stabilization of the GA mRNA and the activated transcription of the PEPCK gene are coordinately regulated within the renal proximal tubule. Therefore, the same signal transduction mechanism is likely to initiate both adaptations. However, the pH-responsive increase in GA mRNA levels in LLC-PK1-FBPase⁺ cells is not blocked by SB-203580 (16). Thus the enhanced binding of ζ-crystallin/NADPH:quinone reductase to the GA mRNA may be mediated by a kinase that is upstream of p38 SAPK (Fig. 3A). This model provides a hypothesis to further characterize the molecular events that allow the renal proximal convoluted tubule cells to sense changes in pH and mediate a response that leads to increased transcription of specific genes and the selective stabilization of certain mRNAs.

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In vitro and in vivo studies of glutamine metabolism in the kidney have shown that glutamine is a major substrate for glutaminase, which catalyzes the conversion of glutamine to glutamate. This reaction is crucial for the regulation of intracellular pH and the generation of glutamate, which serves as an excitatory neurotransmitter.


