Effect of pH and Bicarbonate on Phosphoenolpyruvate Carboxykinase and Glutaminase mRNA Levels in Cultured Renal Epithelial Cells*

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A gluconeogenic strain of renal epithelial cells (LLC-PK₁-F⁺) was used to characterize the effect of pH and bicarbonate concentration on the levels of phosphoenolpyruvate carboxykinase (PCK) and glutaminase (GA) mRNAs. The levels of both mRNAs are markedly dependent upon medium glycose concentration. The level of PCK mRNA is increased with increasing glucose concentration from 0 to 40 mM, whereas the level of GA mRNA is maximal between 3 and 5 mM glucose. When LLC-PK₁-F⁺ cells are grown with 5 mM glucose and then subjected to an acute decrease in pH (from 7.4 to 6.9) and bicarbonate concentration (from 25 to 10 mM), the level of PCK mRNA exhibits a biphasic response. The PCK mRNA is initially increased 4-fold within 3 h, then decreases slightly and subsequently increases between 10 and 20 h to a level that is 17-fold greater than normal. Only the initial increase paralleled the changes observed in vivo. In contrast, after onset of acidosis, the level of GA mRNA initially remains unchanged, is then increased 8-fold between 10 and 16 h, and then decreases slightly. This response closely mimics the results obtained in vivo. A decrease in media pH at constant bicarbonate causes a marked increase in both mRNAs. However, the levels of the two mRNAs are also elevated by decreasing bicarbonate at a constant pH. Thus, both parameters independently affect the level of the two mRNAs. The use of actinomycin D to measure the half-lives of PCK and GA mRNAs at pH 7.4 and 6.9 indicates that stabilization may fully account for the induction of GA mRNA and contributes to the inductive effects of decreased pH and/or bicarbonate on PCK mRNA. Following recovery from acidic conditions, the two mRNAs exhibit a rapid and coordinate decrease (t₁/₂ ~ 20 min). Dexamethasone had no effect on the level of either mRNA, whereas cAMP increased only PCK mRNA. The latter effect was additive with the increase caused by decreased pH and/or bicarbonate and was reversed by incubating in alkalotic media. Thus, the induction of PCK and GA mRNAs during acidosis is initiated in direct response to a decrease in extracellular pH and/or bicarbonate.

As part of the compensatory response to metabolic acidosis, the mitochondrial glutaminase and cytosolic phosphoenolpyruvate carboxykinase activities are increased within the proximal convoluted tube of the rat kidney (1, 2). The increases are due to enhanced rates of synthesis (3, 4) that correlate with elevated levels of the respective mRNAs (5, 6). The studies described in the preceding paper (7) demonstrate that the initial increase in PCK mRNA results from increased transcription. In contrast, GA mRNA levels are increased following a significant lag and occur without stimulating transcription. The adaptations in renal gene expression and glutamine utilization are coordinated with an increase in glutamine synthesis that occurs in liver and muscle (8, 9). Therefore, the adaptive responses in multiple organs could be initiated and coordinated by a humoral factor. Alternatively, the responsive tissues could contain proteins that sense changes in pH and bicarbonate concentration and initiate a series of reactions that mediate and coordinate multiple responses.

A more thorough characterization of the mechanism that mediates the effects of acidosis required the identification of a renal cell line that expresses both mRNAs and that exhibits the responses observed in vivo. In this study we have characterized the effects of medium pH and bicarbonate concentration on the levels of PCK and GA mRNAs expressed in LLC-PK₁-F⁺ cells, a gluconeogenic line of renal proximal tubular epithelial cells (10). The observed responses are similar to the adaptations observed in rat kidney following acute onset or recovery from acidosis (7). Thus, the renal response to acidosis is not likely to be mediated by a humoral factor. In addition, the LLC-PK₁-F⁺ cells should provide an appropriate system to further characterize the molecular mechanism which regulates the adaptations in renal gene expression that occur in response to altered acid-base balance.

EXPERIMENTAL PROCEDURES

Materials–The LLC-PK₁-F⁺ cells were obtained from J. Handler (National Institutes of Health) (10). [α-³²P]dCTP (specific activity, 3000 Ci/mmol) was from Amersham Corp., and GeneScreen was from Du Pont-New England Nuclear. CPCAmp was purchased from Boehringer Mannheim, and dexamethasone was obtained from Anthony Products. The oligolabeling kit was from Pharmacia LKB Biotechnology. AG501-X8 resin and sodium dodecyl sulfate were from Bio-Rad; agarose was from FMC. CH₃H₂O₃H₂O was purchased from Alfa Products. Guanidinium thiocyanate and sodium N-lauroylsarcosine were from Fluka. HA-1004 was from Seikagaku Kogyo. All other chemicals were purchased from Sigma or Fisher. The pPCK-10 and pHF3A-1 plasmids were obtained from R. Hanson (Case Western Reserve University School of Medicine) (11, 12) and L. Kedes (Stanford University School of Medicine) (13), respectively. The pGA-13 plasmid which contains 2.1 kilobases of GA cDNA was obtained by using the original pGA-1 and pGA-2 plasmids (14) to screen a rat kidney Agt10 library.

The abbreviations used are: PCK, phosphoenolpyruvate carboxykinase; GA, glutaminase; CPCAmp, 8-(4-chlorophenylthio)-adenosine-3′:5′-cyclic monophosphate; TSE-1, tissue-specific extinguisher-1.
Cell Culture—LLC-PK-F+ cells were grown to confluence on 100-mm plates using a 50:50 mixture of Dulbecco's modified Eagle's and Ham's F-12 nutrient solutions containing 10% (v/v) fetal bovine serum, 5 mM glucose, 25 mM sodium bicarbonate and adjusted to pH 7.4. The cells were maintained at 37 °C in an atmosphere of 95:5% air:CO2. For experiments in which the effects of bicarbonate concentration and pH were tested independently, media containing the desired bicarbonate concentration and pH were tested independently, media containing the desired bicarbonate concentration was adjusted to the appropriate pH by setting the pCO2 (between 0.5 and 20%). The effect of dexamethasone was determined using cells that were maintained in serum-free medium for 24 h prior to the addition of the glucocorticoid.

Northern Analysis—Isolation of total cellular RNA was generally performed using the cells from a single plate. The monolayer was scraped with a guanidinium thiocyanate solution (15), and the resulting suspension was homogenized for 20 s with a Polytron blender. Phenol/chloroform/isoamyl alcohol extraction of the homogenate and ethanol precipitation of the RNA were performed as described (16). Two additional precipitations in 75 and 100% ethanol were carried out, and the RNA was lyophilized for 30 min, dissolved in diethyl pyrocarbonate-treated water at a concentration of 2–3 µg/µl and stored at −70 °C.

Formaldehyde-agarose gel electrophoresis and Northern blot hybridization were carried out as described previously (16). Slot blot experiments were performed using a Schleicher and Schuell Minifold II manifold. The probes used were the 1.6-kilobase BgII fragment of the pPCK-10 plasmid, which encodes for the cytosolic phosphoenolpyruvate carboxykinase (12) and the 2.0-kilobase Sscl fragment of the pGA-13 plasmid. The blots were hybridized overnight in 50% deionized formamide, 0.25 M Na2HPO4, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 7% sodium dodecyl sulfate, and 100 µg/ml salmon sperm DNA containing 2 x 10^6 cpm of 32P-labeled probe/ml and then washed at high stringency (17). All blots were stripped by washing at 80 °C with 2% glycerol and were rehybridized with a human β-actin probe, pHA-1. Levels of β-actin mRNA were unaffected by the various experimental conditions. Quantification of PCK, GA, and β-actin mRNA levels was accomplished by densitometer scanning of suitably exposed autoradiograms using a QuantaQ QX-7 image processor. The levels of PCK and GA mRNA were standardized relative to those of β-actin mRNA.

RESULTS

Using the procedures described in this study, the presence of a PCK mRNA was not detectable in 10 µg of total RNA isolated from LLC-PK1, OK, or NRK-52E cells, which are established lines of proximal tubule cells derived from pig, opossum, and rat kidney, respectively. In addition, no GA mRNA could be detected in either the LLC-PK1 or NRK-52E cells. Isolated rat renal proximal tubules contain high levels of PCK mRNA, but expression of this gene is rapidly repressed when the cells are maintained in primary culture (18). The confluent primary cultures retain high levels of GA mRNA but fail to exhibit an adaptation in response to altered pH. In contrast, LLC-PK-F+ cells, a line of LLC-PK cells that were selected for their ability to grow in the absence of glucose (10), express both PCK and GA mRNAs (Fig. 1). The levels of the two mRNAs contained in total RNA isolated from cells grown under standard conditions are 10–50-fold lower than that measured in rat kidney. However, when the cells were grown for 24 h in medium containing 10 mM bicarbonate at pH 6.9, the levels of PCK and GA mRNAs were increased 17- and 4-fold, respectively. In contrast, both mRNAs are increased 6-fold in the kidneys of chronically acidic rats (7). The greater increase in PCK mRNA in the LLC-PK-F+ cells could be due to the ability to sustain large decreases in pH and bicarbonate concentration. Conditions which produce similar changes during acute acidosis in vivo are partially compensated during chronic metabolic acidosis.

Effect of Glucose Concentration—PCP is the rate-limiting enzyme for renal gluconeogenesis, and oral administration of glucose has been shown to suppress renal PCK mRNA expression (19). Therefore, the effect of medium glucose concentration on the levels of PCK and GA mRNAs in LLC-PK-F+ cells was assessed. Surprisingly, the level of PCK mRNA increased with increasing glucose concentrations from 0 to 40 mM (Fig. 2). GA mRNA levels also increased with glucose concentrations up to 5 mM but then decreased at higher concentrations. The pronounced effect of glucose was maintained in cells grown under acidic conditions (Fig. 1). As a result the effect of decreased pH and bicarbonate concentration was evident in cells grown either with or without glucose.

Kinetics of Induction of PCK and GA mRNAs—All cells were grown to confluence in medium containing 25 mM bicarbonate at pH 7.4 and then maintained in acidic medium (pH 6.9, 10 mM HCO3−) for various times (Fig. 3). The induction of PCK mRNA showed a biphasic response with an initial 4-fold increase which was followed by a slight decrease and a second increase which reached a plateau of a 17-fold induction after 18 h. After an apparent lag of 8 h, the level of GA mRNA increased 8-fold by 16 h and then decreased to a level that was 4-fold greater than normal. In contrast, the
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level of β-actin mRNA remained unchanged when the cells were transferred to acidic medium (Fig. 1). Whereas only the initial phase of the PCK mRNA response paralleled the adaptations observed in vivo, the adaptation in GA mRNA closely mimicked the in vivo response (7). Thus, the cultured cells should provide an adequate system to study responses of PCK and GA mRNAs to physiological stimuli. Further, the similarity in lag phase and rate of increase between the GA mRNA and the second phase of PCK mRNA induction suggests that the latter responses may be mediated by a common mechanism.

**PH and Bicarbonate Dependence**—The following experiments were designed to determine whether pH and bicarbonate exert independent effects on PCK and GA mRNA levels. Both parameters were varied separately by making appropriate adjustments of the pCO_2_. Fig. 4 illustrates the pH dependence of PCK and GA mRNA levels at constant bicarbonate concentrations of 25 and 10 mM. It is apparent that at a normal bicarbonate concentration both PCK and GA mRNA are increased significantly only at a pH less than 7.1. The mRNA levels are decreased further at alkalotic pH values. However, at a bicarbonate concentration of 10 mM both mRNAs are increased 10- and 6-fold at pH 7.4, respectively. Interestingly, the greatest increase in PCK and GA mRNAs at 10 mM bicarbonate occurs around pH 7.4. Thus, the modulating effect of bicarbonate seems to be greatest within the region of physiological pH regulation. The separate effect of 5-50 mM bicarbonate at constant pH values of 7.4 and 6.9 is illustrated in Fig. 5. The greatest differential effect of bicarbonate occurs at pH 7.4. However, even at pH 6.9 a decrease in bicarbonate from 30 to 10 mM still produces a 2-fold induction of both PCK and GA mRNA. Furthermore, an inhibiting effect on PCK and GA mRNA levels is apparent either by increasing pH or bicarbonate concentration.

**Effect of Acidosis on mRNAs Half-life**—LLC-PK-F* cells were maintained under normal or acidic conditions for 24 h after which the RNA synthesis inhibitor, actinomycin D, was added and the decreases in PCK and GA mRNAs relative to that of β-actin were determined (Fig. 6). In all cases, the observed decreases occurred with first order kinetics. The calculated half-lives for PCK mRNA at pH 7.4 and 6.9 are 3 and 9 h, respectively. The corresponding half-lives for GA mRNA are 2.5 and 15 h, respectively. Thus, stabilization can account for the induction of GA mRNA and may contribute to the induction of PCK mRNA that occurs in LLC-PK-F* cells grown under acidic conditions. Such a stabilization has already been shown to play a significant role in the induction of liver PCK mRNA by cAMP (20) and by glucocorticoids (21).

**Recovery from Acidosis**—When cells were maintained under acidic conditions for 24 h and then returned to normal pH and bicarbonate concentration, the levels of PCK and GA mRNAs returned to normal within 8-10 h (Fig. 7). The decrease occurred with an apparent half-life of 20-30 min for both mRNAs. This calculated half-life correlates well with the t_0 of 30 min obtained for liver PCK mRNA (20, 22). However, this process occurs more rapidly than the apparent half-life of PCK mRNA measured with actinomycin D. Thus selective inactivation may also contribute to the rapid disappearance of the two mRNAs during recovery. Alternatively, the estimation of mRNA half-lives with actinomycin D may be erroneously high, since this drug has been shown to affect mRNA stability (23, 24). Fig. 7 also shows that increasing the pH from 6.9 to 7.4 while maintaining the bicarbonate concentration at 10 mM results in a decreased rate of PCK mRNA decay (t_0 = 1 h) and the establishment of a higher steady...
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Fig. 6. Effect of acidosis on mRNA half-life. Disappearance of PCK and GA mRNAs were observed under normal and acidic conditions in the presence of actinomycin D (0.5 µg/ml). Cells were grown for 24 h under normal or acidic conditions, after which actinomycin D was added. Cells were harvested at the indicated time points after actinomycin D addition. Values represent the means ± S.D. from three different experiments and are expressed relative to the mRNA level before adding actinomycin D.

Fig. 7. Time course of the decrease of PCK and GA mRNA levels following recovery from acidosis. Cells were maintained under acidic conditions for 24 h and then the medium was adjusted to pH 7.4, and the bicarbonate concentration was changed to 25 or 10 mM. Values are the means ± S.D. from three different experiments and are expressed relative to the level prior to recovery, which was taken as 100.

state level. Thus, the bicarbonate concentration may either modulate the selective inactivation of the mRNAs during recovery from acidosis or contribute to the stabilization of the mRNA.

Effect of Glucocorticoids and Cyclic AMP—Treatment with glucocorticoids or various cAMP analogs causes a significant increase in the transcription of PCK mRNA in rat kidney (25). However, the addition of dexamethasone had no effect on PCK or GA mRNA levels in cells grown under either normal or acidic conditions (Fig. 8). In contrast, the addition of CPMAMP produced a large effect on PCK mRNA levels (Fig. 8), resulting in a 7-fold induction at pH 7.4. The combined effects of growth under acidic conditions and the addition of cAMP analog produced a 30-fold increase in PCK mRNA. Thus the effects of cAMP and of decreased pH and bicarbonate concentration are additive. When cells were maintained with saturating cAMP levels for 24 h, the subsequent shift to acidic conditions produced a 4-fold further induction in PCK mRNA (data not shown). In contrast, the subsequent shift of cAMP-stimulated cells to alkalotic conditions (pH = 8.2 and [HCO₃⁻] = 30 mM) caused a 2-fold decrease in PCK mRNA. Furthermore, the cAMP-dependent protein kinase inhibitor, HA-1004, completely inhibited the inductive effect of cAMP but had no effect on the adaptation caused by decreased pH and bicarbonate. CPMAMP also has no effect on GA mRNA levels. Thus, the effect of acidic conditions on the two mRNAs is not mediated by this second messenger.

DISCUSSION

Acute alterations in acid-base balance produce significant changes in the levels of PCK and GA mRNAs within the rat kidney (7). The acute increase in PCK mRNA results from increased transcription, whereas the subsequent increase in GA mRNA occurs without altering its rate of transcription (7). Thus, the adaptation in the two mRNAs is temporally coordinated but occurs through different mechanisms. The adaptive changes in rat kidney are similar to those observed in LLC-PK-F⁺ cells that are subjected to acute changes in medium pH and bicarbonate concentration. In rat kidney, the level of glutamate dehydrogenase mRNA is increased 3-fold during metabolic acidosis. The adaptations observed in response to acute onset and recovery from acidosis occur with kinetics identical to that observed for GA mRNA. Preliminary experiments demonstrate that the levels of glutamate dehydrogenase mRNA contained in LLC-PK-F⁺ cells are also increased in response to growth in acidic medium. This response occurs with kinetics that closely mimic those ob-

² J.-J. Hwang, C. Banner, and N. P. Curthoys, manuscript in preparation.

³ S. Kaiser and T. Welbourne, unpublished data.
erved in vivo. The ability to reproduce the renal responses in cell culture indicates that the in vivo effect of acidosis must be mediated by a cellular mechanism that senses alterations in pH and/or bicarbonate and not by a humoral factor. Transmission of the initial signal could subsequently activate the separate mechanisms that are responsible for the induction of specific mRNAs.

Increasing concentrations of glucose also cause an increase in the PCK mRNA levels in LLC-PK-F+ cells. This result is in contrast to the observation that oral feeding of glucose reduced PCK mRNA levels in rat kidney (19). Similarly, PCK mRNA levels in hepatoma cells are decreased by increasing the medium glucose concentration (26). Furthermore, expression of the hepatic PCK gene is strongly regulated by the counteracting effects of insulin and glucagon (27, 28). However, neither hormone has been demonstrated to affect renal PCK gene expression. This observation is consistent with the concept that renal gluconeogenesis is tightly coupled to amoniagenesis and acid-base balance rather than extracellular glucose homeostasis (4). The mechanism by which glucose affects the levels of PCK and GA mRNAs in LLC-PK-F+ cells remains unknown. It could activate a positive transcription factor or negatively affect expression of a specific extinguisher.

Both the kinetics and the magnitude of the changes in GA mRNAs observed in LLC-PK-F+ cells closely reproduce the adaptation observed in rat kidney (7). The only difference in the two systems is the more rapid decrease in GA mRNA that occurs in the LLC-PK-F+ cells following transfer from acidic to normal medium. However, the acute change in media is likely to produce a more rapid alteration in intracellular pH and bicarbonate concentration than that produced by stomach loading a chronically acidic rat with NaHCO3. Thus, the two systems are very likely to utilize an identical mechanism to regulate GA gene expression.

Of the various primary cultures and established renal cell lines tested, only the LLC-PK-F+ cells expressed detectable levels of PCK mRNA. The F+ line of LLC-PK cells also exhibits an increased level of fructose 1,6-bisphosphatase activity (19) and of GA mRNA. The pleiotropic difference between the selected cells and the parent line suggests that the LLC-PK-F+ cells did not result from a single mutation within the PCK promoter. In contrast to the 2-fold effect observed in rat kidney (7), the level of PCK mRNA in LLC-PK-F+ cells is increased 7-fold by addition of CAMP, an effect that is independent of alterations in pH and bicarbonate concentration. The fusion of minimally differentiated hepatoma cells with fibroblasts results in the repression of various liver genes including PCK (29). This effect is due to a genetic locus termed tissue-specific extinguisher-1 (TSE-1). The residual PCK expression detected in monochromosomal hybrids containing TSE-1 is induced dramatically by CAMP (29, 30). Thus, increased expression of TSE-1 could account for the loss of PCK expression observed in primary cultures and established lines of renal proximal tubular epithelial cells. An altered expression of TSE-1 could account for the pleiotropic changes that are characteristic of the F+ line of LLC-PK cells and the observed effect of CAMP on the levels of PCK mRNA.

An acute decrease in medium pH and bicarbonate concentration causes a biphasic increase in the level of PCK mRNA in LLC-PK-F+ cells. Only the initial phase of this response occurs with kinetics that are similar to the acute increase in PCK mRNA observed in vivo. The adaptive increase in rat renal PCK mRNA is due to increased transcription (7). The subsequent and more pronounced increase in the level of PCK mRNA within LLC-PK-F+ cells occurs with kinetics similar to the increase in GA mRNA. The apparent half-life of the PCK mRNA in LLC-PK-F+ cells is increased 3-fold by growth in acidic medium. Preliminary experiments indicate that expression of a -490-base pair PCK promoter-CAT construct (31) transfected into LLC-PK-F+ cells is increased 2-3-fold by growth in acidic medium. Thus, increased PCK mRNA apparently results from both increased stability and increased transcription. In contrast, the observed change in GA mRNA stability is sufficient to account for its induction.

Treatment with dexamethasone had no effect on the level of PCK mRNA in LLC-PK-F+ cells. The promoter of the rat PCK gene contains multiple glucocorticoid regulatory elements, and the level of PCK mRNA in rat kidney is increased 4-fold by dexamethasone. Thus, the LLC-PK-F+ cells may lack glucocorticoid receptors or the pig PCK gene may not contain a glucocorticoid response element. Alternatively, the absence of a glucocorticoid response could be due to putative upstream repressor elements within the PCK promoter (32), which might be more active in the cultured cells than in rat kidney. This hypothesis is supported by the observation that dexamethasone exerts an effect on PCK mRNA in LLC-PK-F+ cells in the presence of cycloheximide. In cell culture it is possible to vary pH or bicarbonate concentration independently. It was thus possible to demonstrate that the two parameters separately affect the levels of PCK and GA mRNAs. At 25 mM bicarbonate, a pronounced increase in PCK and GA mRNA was observed only at pH values of less than 7.1. In contrast, at 10 mM bicarbonate a significant increase in both mRNAs could be detected even at slightly alkaline pH values. Acute acidosis produces a pronounced decrease in both pH and bicarbonate concentration. Thus, the decrease in both parameters could potentiate the induction of specific mRNAs. However, in chronic acidosis, systemic pH is largely compensated and only plasma bicarbonate concentration is still reduced. Thus, the independent effect of bicarbonate may be essential to maintain the increased synthesis of the GA and PCK enzymes. The observed pH and bicarbonate dependence also indicates that acidosis not only induces, but also represses the levels of PCK and GA mRNAs. Furthermore, the similarity between the effects of pH and bicarbonate on the levels of PCK and GA mRNAs supports the hypothesis that adaptations in the two mRNAs are initiated in response to a common signal. LLC-PK-F+ cells may contain a unique transcription factor whose conformation is altered by slight changes in pH and/or bicarbonate concentration. The conformation favored by acidosis may exhibit increased binding to a specific cis-regulatory element within the PCK promoter. Such a pH dependence has been demonstrated for the interaction between the human glucocorticoid receptor and its cis-acting DNA sequence (33). The LLC-PK-F+ cells should provide a system that will make feasible the further characterization of the mechanism by which renal cells sense alterations in acid-base balance and transduce this information to alter specific gene expression.

REFERENCES
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