Differential regulation of branched-chain α-ketoacid dehydrogenase kinase expression by glucocorticoids and acidification in LLC-PK1-GR101 cells

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Submitted 25 August 2003; accepted in final form 6 November 2003

Wang, Xiaonan, and S. Russ Price. Differential regulation of branched-chain α-ketoacid dehydrogenase kinase expression by glucocorticoids and acidification in LLC-PK1-GR101 cells. Am J Physiol Renal Physiol 286: F504–F508, 2004. First published November 11, 2003; 10.1152/ajprenal.00296.2003.—Acidosis and glucocorticoids (GC) are two catabolic signals associated with chronic renal disease. Previously, we reported that these signals stimulate branched-chain amino acid (BCAA) oxidation in renal tubule cells by increasing both the amount and activation state of branched-chain α-ketoacid dehydrogenase (BCKD). Activation of the BCKD complex could result from decreased expression of BCKD kinase, which inhibits BCKD by phosphorylating its E1α subunit. To investigate this possibility, we examined how dexamethasone and acidification (pH 7.0) influence BCKD kinase expression in LLC-PK1-GR101 cells. Dexamethasone, a synthetic GC, decreased BCKD kinase protein by 65% (P < 0.05 vs. control), whereas a low pH (i.e., pH 7.0) decreased the amount of kinase by 71% (P < 0.05 vs. control). Either GC or acidification reduced BCKD kinase mRNA by 46% (P < 0.05 vs. control), but the two signals together did not reduce kinase mRNA more than either signal alone. To examine the mechanism(s) leading to lower kinase mRNA, kinase transcription was evaluated by transiently transfecting LLC-PK1-GR101 cells with BCKD kinase promoter-luciferase mini-genes containing ∼3.5 kb of proximal rat kinase promoter. GC, but not acidification, decreased luciferase activity 42% (P < 0.05 vs. control). Nuclear run-on assays confirmed that GC decrease kinase mRNA by attenuating its transcription. Thus two catabolic signals associated with renal failure, GC and acidification, reduce BCKD kinase expression by different mechanisms. These responses lead to an increase in the activation state of BCKD and a resulting acceleration of BCAA degradation.

branched-chain amino acids; acidosis; gene transcription

THE ESSENTIAL BRANCHED-CHAIN amino acids (BCAA), especially leucine, serve as important regulators of protein homeostasis by influencing the rates of protein synthesis and degradation (20, 26). They can also influence intracellular signaling (e.g., mTOR) and apoptosis in some cell types (19, 27). Generally, BCAA are conserved for new protein synthesis, but in some catabolic conditions, adaptive responses that maintain the intracellular levels of these amino acids appear impaired. For example, in patients with chronic kidney disease (a condition that stimulates whole body and muscle protein degradation), plasma and intracellular levels of BCAA in muscle are low (3) but valine decarboxylation in muscle of experimental animals was stimulated by chronic renal insufficiency (13). Branched-chain ketoacid dehydrogenase (BCKD) is the mitochondrial enzyme that catalyzes the irreversible oxidative decarboxylation of the BCAA. Normally, its activity is low when BCAA are limited and stimulated when BCAA are high (14). In contrast to chronic renal failure, plasma levels of BCAA were high in acute diabetes (another catabolic state associated with muscle wasting) and BCKD activity was accelerated in muscle (1). Based on these and other studies of rats with various catabolic conditions, it is evident that conclusions about the status of BCAA degradation in cells cannot be made based on circulating concentrations of BCAA alone.

BCDK activity in tissues can be regulated in several different ways. In some instances, changes in measured BCKD activity correlate with the levels of BCKD subunits (5, 35). It has also been speculated that processing of BCKD subunits and assembly of the enzyme complex may be regulated. Finally, the activity of BCKD can be altered by changing the phosphorylation state of the E1α subunit (33). BCKD kinase is a unique nuclear-encoded, mitochondrial kinase that inhibits the activity of BCKD by phosphorylating Ser-292 and Ser-302 of the BCKD E1α subunit (31, 36). BCKD can also be activated by a poorly characterized phosphatase. The amount of active BCKD complex varies widely in different organs and is determined by the balance between the phosphatase and BCKD kinase activities. BCKD is almost completely active in liver, but in muscle, only ∼5% of BCKD is in the active state (4); in other organs, BCKD activity is partially active (e.g., ∼60–70% active in kidney). The ability to modulate the activation state of BCKD provides mechanisms for 1) increasing BCKD activity to provide energy precursors (i.e., carbon chains for gluconeogenesis) when other energy sources are limited in cells and 2) decreasing BCKD activity to maintain the levels of BCAA when sources (e.g., diet) are restricted.

Efforts to elucidate the biochemical mechanisms of BCAA metabolism have been hampered by a poor understanding of the physiological signals that regulate BCKD activity. In earlier studies, we demonstrated that acidosis and glucocorticoids, two catabolic signals associated with chronic kidney disease, increase BCKD activity in renal tubule cells and skeletal muscle (9, 32, 35). To examine how these signals increase BCKD activity, we studied BCKD in LLC-PK1-GR101 cells [renal tubule cells that we engineered to express glucocorticoid receptors (35)]. One response to these stimuli was increased expression of the BCKD E1α and E2 subunits (34), but the activation state of BCKD was also increased by these signals (35). Others reported that short-term regulation of BCKD activity can result from a change in BCKD kinase content, but these studies provided few insights about the mechanisms regulating BCKD kinase (8, 17, 23, 29). The goal of our studies was to determine whether glucocorticoids and acidification alter BCKD kinase gene expression in LLC-PK1-GR101 renal tubule cells and to determine how the responses were achieved.

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Materials and Methods

Materials. DMEM, penicillin, streptomycin, trypsin, EDTA, and glutamine were from Gibco BRL (Grand Island, NY); FBS from Irvine Scientific (Santa Ana, CA); dexamethasone (Dex) from Elkins-Sinn (Cherry Hill, NJ); hygromycin from Calbiochem-Novabiochem (La Jolla, CA); Hybond enhanced chemiluminescence (ECL) membranes, Western blot reagents, and [α-32P]UTP and [α-32P]dCTP from Amersham (Arlington Heights, IL); Zeta Probe GT Nylene membranes from Bio-Rad Laboratories (Hercules, CA); calcium phosphate transfection kits from Life Technologies (Gaithersburg, MD); and pRL-TK and reagents for the Dual-Luciferase reporter assay were from Promega (Madison, WI). Other chemicals were from Sigma (St. Louis, MO).

Cell culture. LLC-PK1-GR101 cells have been described and were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 60 IU/ml penicillin, 60 μg/ml streptomycin, and a 10% CO2 atmosphere at 37°C. Hygromycin (0.8 mg/ml) was also added to the growth medium to maintain expression of the human glucocorticoid receptor. Cells (1 x 106) were plated in 60-mm plastic dishes for transfection experiments and were used when they reached 70% confluence. For all other experiments, cells were plated in T-75 flasks and were used at confluence. To acidify the medium, HCl was added to achieve pH 7.0 after equilibration with 10% CO2.

Measurement of mitochondrial BCKD kinase protein. Mitochondrial proteins were prepared from LLC-PK1-GR101 cells (12). Proteins (20 μg) were separated in 10% SDS-polyacrylamide gel and were transferred to a polyvinylidene difluoride membrane. BCKD kinase protein was detected with polyclonal BCKD kinase antisera (kindly provided by Dr. D. J. Danner, Emory University) using a chemiluminescence detection system (Amersham ECL kit). Multiple exposures were made to ensure that band intensities did not exceed the linear detection range of the film. Band intensities of the immunoreactive proteins were measured with a Bio-Rad densitometer. The antisera reacted nonspecifically with an unknown (~58-kDa protein in the renal cell lysate, but its abundance was not changed by either acidification or Dex.

RNA isolation and analysis. Total RNA was isolated using TriReagent, separated by electrophoresis in a 1% agarose/formaldehyde gel, and transferred to Zeta Probe GT nylon membranes. Ribosomal RNAs were visualized by staining with methylene blue. Afterwards, Northern blot hybridizations were performed with a rat BCKD kinase cDNA in a solution containing 5× SSC, 5 Denhardt’s solution, 7% SDS, deionized formamide, 10% polyethylene glycol, and 50 μg/ml denatured herring testis RNA at 42°C overnight. Subsequently, membranes were washed once with 2× SSC/0.5% SDS at 60°C for 20 min each. Autoradiographic signals were quantified by densitometric analysis and expressed relative to the corresponding 28S RNA value.

Transcription assay. Cells were washed with ice-cold PBS three times and scraped in inharvest buffer composed of 10 mM HEPES (pH 7.5), 150 mM KCl, 4 mM magnesium acetate, 0.5% IGEPAL CA-630, and 5 mM β-mercaptoethanol. Nuclei were isolated as described (2). Run-off assays were performed by suspending the isolated nuclei in a buffer containing 3.3 mM KCl, 6.1 mM DTT, 25 mM MgCl2, 0.6 mM ATP, 0.3 mM CTP, 0.3 mM GTP, 40 U RnAsin, and 250 μCi[α-32P]UTP (3000 Ci/mmol) and incubating the suspension for 30 min at 25°C. The newly transcribed [32P]-labeled RNA was purified using TriReagent and hybridized with immobilized cDNA probes (2 μg each) for rat GAPDH, human γ-actin, and rat BCKD kinase. To provide a negative hybridization control, linearized pGEM3zf (Promega) was included. Hybridizations were performed in 0.12 M Na2HPO4, 0.25 M NaCl, ×5 Denhardt’s, 50% formamide, 10% SDS, 50% polyethylene glycol, and 50 μg/ml denatured herring DNA at 47°C for 3 days. Subsequently, the membranes were washed with 25 μg/ml ribonuclease A for 30 min at room temperature followed by ×1 SSPE/0.5% SDS at 60°C for 15 min.

Transient transfection and luciferase assays. Transient DNA transfection of LLC-PK1-GR101 cells was accomplished using 6 μg of total DNA (3 μg BCKD kinase promoter-fluorescent luciferase reporter gene plasmid, 0.5 μg pRL-TK Renilla luciferase transcription control vector (Promega), and 2.5 μg carrier DNA) per 60-mm dish as described (34). BCKD kinase promoter-fluorescent luciferase reporter plasmids containing portions of the rat BCKD kinase promoter from −3500 to +264 or shorter (15) were a gift from Dr. D. T. Chuang (University of Texas Southwestern Medical Center). Cells were transiently transfected using the calcium phosphate transfection method and were subsequently maintained in DMEM supplemented with 10% charcoal-treated FBS (which lacks endogenous steroids) for 72 h. During the final 24 h of the experiment, cells were incubated with medium containing Dex (50 nM) or acidified medium (pH 7.0). Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) and a Turner TD-20/20 luminometer. Results of the firefly luciferase activities were normalized for differences in transfection efficiency using the respective Renilla luciferase activities.

Statistical analyses. Data are presented as means ± SE. Results for two treatment groups were analyzed using the unpaired Student’s t-test. Comparisons between three or more treatment groups were performed by one-way analysis of variances followed by a pairwise post hoc comparison using the Student-Newman-Kuels method. Outcomes were considered significant when P < 0.05.

Results

Acidification or Dex decreases the BCKD kinase protein. We previously reported that extracellular acidification or glucocorticoid increases BCKD activity in LLC-PK1-GR101 cells, in part, by increasing the amount of BCKD in the active state. To examine whether these responses were due to a change in BCKD kinase expression, we performed a Western blot analysis of mitochondrial proteins from LLC-PK1-GR101 cells that had been incubated at pH 7.4 or 7.0 with or without 50 nM Dex for 24 h. All studies were performed with cells maintained in medium supplemented with serum-treated charcoal to remove endogenous glucocorticoids. When cells were acidified or incubated with Dex, immunodetectable BCKD kinase was decreased 71% (P < 0.05 vs. control) or 65% (P < 0.05 vs. control), respectively (Fig. 1). Incubation in acidified medium supplemented with Dex also significantly decreased the amount of kinase protein, relative to the level in control cells, but the combined effects were not greater than either individual response alone (Fig. 1). These results are evidence that both glucocorticoids and acidification increase BCKD activity by reducing the amount of BCKD kinase that is available to phosphorylate (i.e., inactivate) the E1α subunit of the BCKD complex.

Regulation of BCKD kinase transcription. To investigate the mechanisms leading to decreased BCKD kinase expression, we evaluated the relative levels of BCKD kinase mRNA in LLC-PK1-GR101 cells by Northern blot analysis. Incubation in acidified (pH 7.0) medium for 24 h produced a decrease in kinase mRNA of 46 ± 7% (P < 0.05 vs. control; Fig. 2). There was a nearly identical decrease (54 ± 9%; P < 0.05 vs. control) in the mRNA after Dex treatment. The combination of both signals decreased kinase mRNA 56 ± 8% (P < 0.05 vs. control), but the extent of the decrease was not significantly greater than with either signal alone.
To determine whether the decrease in kinase mRNA resulted from decreased gene transcription, we performed a series of transfection studies with rat BCKD kinase promoter-luciferase reporter gene plasmids. Cells were transiently transfected with BCKD kinase promoter-luciferase minigene plasmids consisting of 3,500, 1,700, or 128 bp of promoter sequence (15); the open arrowhead (NS) designates the position of a nonspecific protein detected with the antibody. The experiment was repeated 3 times with similar outcomes. The kinase bands were quantified by densitometry, and the combined results are reported graphically (bottom) as the mean percentage ± SE (n = 6/treatment group) of the control group average. CTL, control cells; GC, Dex-treated cells; H+, acidiﬁed cells; GC/H+, acidiﬁed, Dex-treated cells. *Signiﬁcant difference (P < 0.05) between the control and experimental treatment group.

Fig. 1. Acidification and dexamethasone (Dex) decrease branched-chain α-ketoacid dehydrogenase (BCKD) kinase protein in renal tubule cells. LLC-PK1-GR101 cells were incubated at either pH 7.4 or 7.0 with or without 50 nM Dex for 24 h. Afterward, mitochondria were isolated and Western blot analysis of mitochondrial proteins (20 kDa) was performed with antisera that recognizes rat BCKD kinase. Top: blot results from 1 representative treatment group. The open arrowhead (NS) designates the position of a nonspeciﬁc protein detected with the antibody. The experiment was repeated 3 times with similar outcomes. The kinase bands were quantiﬁed by densitometry, and the combined results are reported graphically (bottom) as the mean percentage ± SE (n = 6/treatment group) of the control group average. CTL, control cells; GC, Dex-treated cells; H+, acidiﬁed cells; GC/H+, acidiﬁed, Dex-treated cells. *Signiﬁcant difference (P < 0.05) between the control and experimental treatment group.

Fig. 2. Effects of acidification and Dex on BCKD kinase mRNA. LLC-PK1-GR101 cells were incubated at pH 7.4 or 7.0 with or without Dex (50 nM) for 24 h. BCKD kinase mRNA was measured by Northern blot analysis. Results are reported graphically (bottom) as the mean percentage ± SE (n = 9/treatment group) of the control group average. The experiment was repeated 3 times with similar outcomes. *Signiﬁcant difference (P < 0.05) between the control and experimental treatment group.

Fig. 3. Dex decreases BCKD kinase promoter activity. LLC-PK1-GR101 cells were transiently transfected with BCKD kinase (3.5 or 1.7 kb) promoter-luciferase minigene plasmids. Cells were incubated at pH 7.4, pH 7.0, or pH 7.4 + 50 nM Dex in 24 h. The ﬁreﬂy luciferase activity in each well was normalized for transfection efﬁciency using the respective Renilla luciferase activity. Normalized luciferase activities in the treatment groups were calculated as the percentage of the mean value of the control group. Results are the mean percentage ± SE (n = 12/treatment) of the control group average. The experiment was repeated 4 times with similar outcomes. *Signiﬁcant difference (P < 0.05) between the control and experimental treatment group.

Fig. 4. Dex decreases BCKD kinase transcription. LLC-PK1-GR101 cells were incubated in control or acididiﬁed media ± Dex (50 nM) for 24 h. Again, LLC-PK1-GR101 cells were maintained in DMEM supplemented with charcoal-stripped serum for 48 h before addition of Dex to ensure that any effect of acididiﬁcation was not due to endogenous steroids. Acididiﬁcation slightly decreased (13% vs. control) the amount of kinase promoter-dependent luciferase activity (normalized for transfection efﬁciency) in cells transfected with the 3,500-bp BCKD kinase promoter minigene, but the decrease was not statistically signiﬁcant (Fig. 3). In contrast, Dex decreased luciferase activity by 42% (P < 0.05 vs. control) in cells transfected with the same minigene, construct (Fig. 3). Acididiﬁcation plus glucocorticoids did not inhibit kinase transcription more than Dex alone. Dex also suppressed kinase promoter activity by 16% (P < 0.02 vs. control cells) in cells transfected with a minigene containing the region of the kinase promoter from −1,700 to +264 (Fig. 3). The partial attenuation of the suppression of kinase promoter activity was reproducible in several independent experiments, suggesting that more than one element in the
kinase promoter is responsive to glucocorticoids. Consistent with this idea, shortening the length of kinase promoter to the region from −128 to +264 abrogated the suppression of kinase transcription by glucocorticoids (data not shown).

Finally, we confirmed that Dex decreases transcription of the BCKD kinase gene by performing nuclear run-off experiments using nuclei isolated from control or Dex-treated cells. As expected, Dex decreased kinase transcription (Fig. 4). This response was specific for the kinase because the amounts of newly transcribed GAPDH and γ-actin mRNAs were unaffected by glucocorticoids.

DISCUSSION

The activity of BCKD is highly regulated in response to nutritional and physiological signals. In many conditions associated with cachexia (e.g., starvation, diabetes, increased cytokines), BCKD activity is increased in several organs (9, 11, 28, 30). Studies examining the mechanisms leading to increased BCKD activity have consistently found either increased production of the complex subunit proteins and/or an increase in the activation state of the enzyme. For example, we demonstrated that glucocorticoids and acidification are signals associated with kidney disease that increase the activation state of BCKD as well as the amount of BCKD proteins in LLC-PK1-GR101 renal tubule cells (35). In the present work, we provide evidence that these signals increase the activation state of BCKD by attenuating the expression of BCKD kinase protein. It was surprising that the suppressive effects of acidification and glucocorticoids were not additive. Considering that the signals appear to act through separate mechanisms, we find this outcome difficult to explain but note that it was consistent in several experiments.

There are a few instances in which hormones have been shown to change BCKD kinase expression in liver cells. Nellis et al. (29) found that insulin increased BCKD kinase mRNA and protein but the molecular basis of the response was not determined. Huang and Chuang (17) reported that glucocorticoids downregulated the kinase mRNA (the effect on kinase protein was apparently not tested). Further experiments indicated that Dex did not alter kinase mRNA stability, nor did it alter the activity of a transfected reporter gene plasmid containing the proximal 3 kb of rat kinase promoter. This led the investigators to conclude that the glucocorticoid-responsive element mediating the suppression of transcription in liver was located upstream of base −3500. In contrast, our data findings indicate that the glucocorticoids suppress BCKD kinase transcription and that there are several response elements located within 3,500 bases of the transcription initiation site.

How could glucocorticoids decrease BCKD kinase transcription in renal tubule cells? Huang and Chuang (15) reported that at least one potential glucocorticoid response element (GRE) is present in the promoter region of the rat BCKD kinase but GREs are typically involved in gene transactivation rather than transrepression. When the mechanisms of glucocorticoid-induced suppression of transcription of other genes have been studied, activated glucocorticoid receptors typically interfere with the binding of other transcription factors (e.g., C/EBP, NF-kB, Sp1), which influence either basal transcription or gene transactivation (7, 21, 22). Moreover, the manner in which transrepression was accomplished was gene specific, in sharp contrast to the way that glucocorticoids increase gene transcription. In the case of BCKD kinase, there are several candidate transacting factors that may regulate its expression, but only one study examined the transcriptional regulation of this gene. Huang and Chuang (16) found that Sp1 is important for basal kinase promoter activity. We think it is unlikely that glucocorticoids decrease kinase transcription by interfering with Sp1 because Dex did not attenuate the reporter activity of the −128/+264 kinase promoter-luciferase reporter plasmid despite the presence of Sp1 binding sites located at −142 to −147 and +31 to +36 in the promoter insert.

Our studies also provide some clues about the way that acidification decreases kinase expression in renal cells. Our data suggest that acidification decreases the stability of kinase mRNA because the amount of BCKD mRNA is reduced even though transcription is unchanged. How could acidification decrease kinase mRNA? We could not find any instances where acidification downregulates a specific mRNA; however, acidification does stabilize glutaminase mRNA in renal epithelial cells (18). Curtoys and Gstraunthaler (6) identified an 8-base adenosine and uridine (AU)-rich sequence in the 3′-untranslated region, which confers sensitivity to a low pH (24). Recently, this same group reported that γ-crystallin/NADPH: quinone reductase binds to this response element and suggested that this interaction initiates the acidification-induced stabilization of glutaminase mRNA (6). It is possible that acidosis could interfere with the interaction between a stabilizing factor and a response element or decrease the expression of a stabilizing factor. Acidification also increases the level of PEPCK mRNA in LLC-PK1-FBPase cells. In this case, acidification increases PEPCK gene transcription (10), but it is notable that an instability element also has been identified in the 3′-untranslated region of the PEPCK mRNA (25). Functional analysis of the stability element has not identified a protein(s) that binds to the site or signals (e.g., acidosis, glucocorticoids, cAMP) that regulate the level of PEPCK mRNA through this site. From these studies, it is clear that there are several mechanisms by which acidification regulates the level of specific mRNAs.

In conclusion, our results demonstrate that glucocorticoids and acidosis increase the activation state of BCKD by decreasing the amount of BCKD kinase. In addition, these signals also increase the expression of BCKD complex proteins. These mechanisms for regulating BCKD activity work in concert to enable renal cells to increase the degradation of the essential BCAA in chronic kidney disease.

ACKNOWLEDGMENTS

The authors thank Drs. D. Danner and D. Chuang for contributions of kinase antibody and kinase promoter constructs, respectively, and Dr. H. Franck for a careful reading of the manuscript.

GRANTS

These studies were supported by National Institutes of Health Grants DK-63658 and 50740 to S. R. Price.

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