Insulin increases branched-chain α-ketoacid dehydrogenase kinase expression in Clone 9 rat cells

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Nellis, Mary M., Christopher B. Doering, Andrea Kasinski, and Dean J. Danner. Insulin increases branched-chain α-ketoacid dehydrogenase kinase expression in Clone 9 rat cells. *Am J Physiol Endocrinol Metab* 283: E853–E860, 2002. First published June 3, 2002; 10.1152/ajpendo.00133.2002.— The branched-chain amino acids (BCAA) are committed to catabolism by the activity of the branched-chain α-ketoacid dehydrogenase (BCKD) complex. BCKD activity is regulated through the action of the complex-specific BCKD kinase that phosphorylates two serine residues in the E1α subunit. Greater BCKD kinase expression levels result in a lower activity state of BCKD and thus a decreased rate of BCAA catabolism. Activity state varies among tissues and can be altered by diet, exercise, hormones, and disease state. Within individual tissues, the concentration of BCKD kinase reflects the activity state of the BCKD complex. Here we investigated the effects of insulin, an important regulator of hepatic metabolic enzymes, on BCKD kinase expression in Clone 9 rat cells. Insulin effected a twofold increase in message levels and a twofold increase in BCKD kinase protein levels. The response was completely blocked by treatment with LY-294002 and partially blocked by rapamycin, thus demonstrating a dependence on phosphatidylinositol 3-kinase and mTOR function, respectively. These studies suggest that insulin acts to regulate BCAA catabolism through stimulation of BCKD kinase expression.

hormone-controlled gene expression; branched-chain amino acids

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THE BRANCHED-CHAIN amino acids (BCAA), leucine, isoleucine, and valine, are classified as essential components of the mammalian diet because they cannot be synthesized de novo. BCAA account for up to 20% of the residues in the average protein. Catabolism of the BCAA provides energy, and their products serve as precursors for fatty acid synthesis. BCAA can modify other metabolic processes through a poorly understood mechanism whereby cells sense the concentration of these amino acids. Protein turnover, especially in the liver and muscles, is slowed when the BCAA concentration is maintained (6, 12, 32, 37, 38).

Another player in the cellular regulation of protein turnover is insulin, which stimulates protein synthesis, especially in liver (11, 15). A combination of insulin and leucine may be needed for the promotion of protein synthesis, and this action may occur through separate pathways to enhance assembly of the translation initiation complex (2, 5, 22). As expected, because tissues respond differently to insulin exposure, tissue-specific variation in these effects is reported (9, 19, 35, 40).

Maintenance of cellular BCAA concentration in mammals results from a balance between supply and catabolic loss. Supply results from dietary intake or breakdown of endogenous protein. Irreversible loss results from catabolism of the BCAA that begins with their reversible transamination to yield the branched-chain α-ketoacids (BCKA). Oxidative decarboxylation of the BCKA commits these compounds to their catabolic fate. The reaction is catalyzed by branched-chain α-ketoacid dehydrogenase (BCKD), a nuclear encoded multienzyme complex present in the mitochondria of all cells (54). Catalytic activity of BCKD requires four different proteins: an E1($\alpha_2\beta_2$) decarboxylase, an E2 dihydrolipoyl transacylase, and an E3 dihydrolipoyl dehydrogenase.

Activity state of BCKD is regulated by the phosphorylation status of the E1α subunit (25, 44). Addition of phosphate to Ser^{332} and Ser^{337} (numbering begins with the initiating methionine in agreement with the adopted rules of nomenclature; Ref. 3) effectively blocks the binding site for the BCKA, thus preventing the overall activity of the complex (1). The amount of BCKD in the unphosphorylated state relative to the total amount of BCKD (unphosphorylated + phosphorylated) represents the BCKD activity state and is expressed as a percentage. Although a putative BCKD phosphatase is reported, the activity state of BCKD is more closely related to the expression of BCKD kinase than to a balance between steady-state activities of the kinase and phosphatase (13, 14, 18). For example, in liver BCKD kinase, protein levels are low, leaving most of the BCKD complex catalytically active. In contrast, BCKD activity state is low in the muscle, where BCKD kinase expression is high (16).

BCKD activity state and, for some conditions, BCKD kinase expression are shown to vary with exercise, diet, and hormonal state (7, 28, 34, 36, 43, 49, 51). These variations are presumed to result from cellular sensing of BCAA concentration in combination with the energy needs of the cell. For example, rats fed low-protein diets demonstrate decreased BCKD activity state in liver compared with rats fed normal or high-protein diets (8, 21, 42). Starvation, however, induces activation of BCKD to provide energy to the body. Energy expenditure induced by exercise is associated with an activation of the BCKD complex in rats and humans (28, 34, 49, 51).

The action of insulin is known to stimulate BCKD activity in adipose tissue by decreasing the phosphorylation state of E1α (20). In diabetic rats, insulin withdrawal resulted in a 50% decrease in liver BCKD kinase protein levels and an increased BCKD activity state (30). Another study with diabetic rats showed that brain BCKD activity state is 1.6-fold higher than that of control rats (10). Collectively, these findings implicate a role for insulin in the regulation of BCAA catabolism through changes in BCKD kinase expression.

We hypothesize that the ability of insulin to reduce proteolysis involves upregulation of BCKD kinase, thereby decreasing the activity state of BCKD and, in turn, BCAA catabolism. This response would increase cellular BCAA levels and help promote protein synthesis. Here we investigate the ability of insulin to regulate the expression of BCKD kinase in rat cells and use inhibitors of insulin signaling to examine the pathways involved. This cell line has been used extensively as a model for hepatocyte function, including insulin-mediated signaling responses (46–48).

METHODS

Cell culture. Rat cells [Clone 9, American Type Culture Collection (ATCC)] were maintained in Ham-s F-12K media (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) in a humidified 5% CO_2 atmosphere at 37°C. Cells used in these studies were between *passages 4* and *8* after retrieval from freeze. The cells were aliquoted into 10-cm plates and grown for 48 h (~80% confluent). Cells were washed twice with PBS, placed in fresh F-12K medium, as described above, without serum for 20 h, and then placed in serum-free F-12K medium containing 100 nM insulin. Unless stated otherwise, all analyses were done 20 h after insulin administration. Inhibitors of the insulin-signaling pathways were used at the following concentrations: LY-294002 (50 μM), rapamycin (200 nM), and PD-098059 (25 μM). Each inhibitor was added 1 h before the addition of insulin. The transcription inhibitor, actinomycin D (1 μg/ml), was added along with insulin.

Mitochondria isolation and Western blot analysis. After harvesting, cells were disrupted by dounce homogenization on ice in a buffer containing 220 mM mannitol, 70 mM sucrose, and 2 mM HEPES-KOH, pH 7.4. Mitochondria were isolated from the homogenate by centrifugation at 500 *g* for 5 min at 4°C to remove nuclei and cell debris, followed by centrifugation of the supernatant at 6,000 *g* for 10 min at 4°C to pellet the mitochondria. Mitochondria were resuspended in the isolation buffer and assayed for protein concentration by use of a bicinchoninic acid (BCA) kit (Pierce). Ten micrograms of mitochondrial protein were resolved in 10% SDS-PAGE and transferred to a Hybond-ECL (Amersham) nitrocellulose membrane for Western blot analysis. The membrane was blocked with a solution containing 50 mM Tris, pH 7.5, 0.88% NaCl, 5 mM EDTA, 0.25% gelatin, and 1% Tween 20 and incubated for 2 h at room temperature with rabbit antisera (1:10,000 dilution) that specifically recognize BCKD kinase (16). Each of five independent Western blots were analyzed first with antisera for BCKD kinase, and then the membrane was stripped by soaking in a solution of 100 mM β-mercaptoethanol, 2% (wt/vol) SDS, and 62.5 mM Tris·HCl, pH 6.7, at 60°C for 30 min. The membrane was then used with antisera that recognize the catalytic subunits of BCKD. Detection of antigenic protein was with the Amersham ECL system according to the manufacturer's instructions. Visualization was either with the Typhoon (Molecular Dynamics) phosphoimager or with exposure to Blue Bio film (Denville). Each membrane was therefore normalized to itself, and the ratio of density of the kinase protein to each of the catalytic subunits was calculated, and treatment conditions were compared with the serum-starved control sample.

Phosphorylation of PKB/Akt. Serum-starved and insulin-treated cells grown in 10-cm plates were harvested with a cell scraper in 600 μl of RIPA buffer [20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 μg/ml leupeptin, and 0.1 μg/ml aprotinin]. Ten micrograms of total cellular protein from insulin-treated and untreated cells were separated by 10% SDS-PAGE. The amount of phosphorylated PKB/Akt present in treated and untreated cells was determined with the use of the phospho-Akt and Akt antibodies (New England Biolabs).

BCKD activity assay. Rat cells were aliquoted into a 24-well tissue culture plate and grown to a confluence of ~80%. Cells were placed in serum-free F-12K media for 20 h and then treated with insulin as above (6 wells/treatment). The BCKD kinase inhibitor α-chloroisocaproate (CIC; 1 mM) was added to three of the six wells for each experimental condition, and the plates were incubated at 37°C for 10 min before initiation of the BCKD assay by addition of [1-^{14}C]leucine (0.5 μCi/reaction) made to 430 μM with unlabeled leucine (33). Incubation with CIC allows endogenous activation of BCKD for assessment of total BCKD activity within the cells. The activity state of the BCKD complex was calculated as the activity (pmol $^{14}CO_2$ released·mg protein^{-1}·3 h^{-1}) in the cells not treated with CIC divided by the activity in the cells treated with CIC (total activity) and is expressed as a percentage.

Northern blot analysis. Total RNA from the rat cells was isolated using Tri-Reagent (Sigma). RNA was treated with a formaldehyde sample buffer (Ambion) and separated by electrophoresis on a 1% agarose-formaldehyde gel. Duplicate samples were loaded onto the gel, and, after electrophoretic separation, one-half of the gel was stained with ethidium bromide for visualization, and the other one-half was used for transfer of the RNA to a MagnaGraph (Micron Separations) nylon membrane (4). Rat BCKD kinase cDNA was labeled by incorporation of α-[^{32}P]dCTP by use of the RediPrime kit (Amersham Pharmacia Biotech), and hybridization was done at 42°C overnight. The membrane was washed three times in 2× standard sodium citrate (SSC)-0.1% SDS and twice with 0.2× SSC-0.1% SDS for 5 min/wash at 42°C. Hybridization of

the probe was detected by autoradiography with the use of Kodak Biomax MS film. The membrane was washed four times with 0.1× SSC-1% SDS-40 mM Tris·HCl, pH 7.6, at 80°C for 5 min to remove the probe. The membrane was then hybridized with a radiolabeled 18S ribosomal RNA probe to assess RNA loading.

Luciferase reporter assay. Fragments of the DNA sequence corresponding to the putative BCKD kinase promoter were inserted into a firefly luciferase reporter plasmid (Promega). One fragment contained 1,700 bp upstream of the transcriptional start site, the second held the first 449 bp upstream, and the third contained the first 128 bp. Individually, these plasmids were transfected into rat cells along with a *Renilla* luciferase control plasmid (Promega) with the use of Fugene 6 (Roche), following the manufacturer's protocol (26). After 6 h, the transfection medium was replaced with serum-free medium. Cells were treated with 100 nM insulin and harvested 20 h later by use of 1× passive lysis buffer (Promega). Luciferase activity was determined with a Turner 20/20 Luminometer using the Dual Luciferase Assay Kit (Promega).

RNA extraction and reverse transcriptase. Total RNA was prepared using the RNeasy kit (Qiagen) from the rat cells incubated in serum-free medium without or with 100 nM insulin for 2 and 20 h. RNA integrity was verified by electrophoresis and ethidium bromide staining, and the concentration was estimated by absorbance at 260/280 nm. The Superscript First Strand Synthesis System (Invitrogen) was used to transcribe 5 μg of total RNA using oligo dT primer according to the manufacturer's instructions.

Real-time PCR. Primers for BCKD kinase cDNA were HPLC purified (forward 5′-GGAGGGCAGGTGAGCTTTT-GTTTC-3′; reverse 5′-GCCTGCCCCATAGTGACCTTTACC-3′) and produced a fragment of 288 bp covering the region from bp 1671 to 1959. Reaction conditions for PCR amplification in the Light Cycler (Amersham) were as follows: 7.2 μl H_2O, 2 μl 10× PCR Buffer II (Roche), and 3 μl $MgCl_2$ (3.75 mM) 1.3 μl each of the forward and reverse primers (0.01 μg/μl), 1 μl dNTP (0.5 mM), 1 μl 10× BSA (New England Biolabs), 1 μl SYBRgreen (Roche), and 0.2 μl Platinum *Taq* DNA polymerase (GIBCO). Light Cycler mastermix (18 μl) was added to glass capillaries, and 2 μl of cDNA (2 and 20 h of both insulin-treated and control samples) were added as a PCR template. The following Light Cycler run protocol was used: denaturation program (94°C for 3 min), amplification and quantification program (94°C for 2 s, 62°C for 10 s, and 72°C for 30 s with a single fluorescence measurement), repeated 35 times. The melting curve program increases the temperature from 68 to 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement ending with a cooling step to 40°C. Values are calculated relative to a standard curve produced by serial dilution of total RNA from untreated cells.

Insulin binding. Insulin binding by these cells was assessed as previously described (31). Briefly, confluent cells at *passages 7* and *18* were incubated for 2 h at 20°C with ^{125}I-labeled insulin (250–350 nCi/ng) in 1 ml of Tris-buffered Earle's balanced salt solution. Cells were then washed 4× with ice-cold saline and solubilized with 0.4 ml of 1 N NaOH and counted with a γ-counter and related to total cellular protein in the reaction. Specific binding was estimated by measuring binding of labeled insulin in the presence of 1 μM unlabeled insulin. The amount of specific binding decreased by 50% in the higher passage cells relative to those at *passage 7* (data not shown).

RESULTS

Under normal physiological conditions, the activity state of the BCKD complex in liver tissue is 60% or greater, implying that BCAA catabolism can readily occur (21). For insulin to promote protein synthesis in liver, BCAA must be available for synthesis of new proteins. Therefore, the activity state of the BCKD complex must be decreased to minimize BCAA catabolism. To test this hypothesis, rat cells were grown in culture and treated with insulin. Activity state of the BCKD complex was determined in cells 20 h after insulin administration. Insulin treatment produced 50% reduction in the activity state (Fig. 1).

Although the activity state in these cultured rat cells is below that reported for liver tissue samples (52), the cells demonstrated an insulin response, the primary requirement for the study. Because total activity remained constant, the change in activity state must reflect an increase in the phosphorylation of the E1α component, thereby reducing the percentage of the complex that is catalytically active. Northern and Western blot analyses demonstrated that the steady-state concentration of mRNA and protein for BCKD kinase was higher in cells treated with insulin than in controls and that this response persisted for at least 20 h (Fig. 2, *lanes 1* and *2*). The averaged ratio of BCKD kinase mRNA to 18S RNA was 2.07 ± 0.50 from four independent determinations. In parallel, the average change in BCKD kinase protein relative to the E1α protein of the BCKD complex is 2.6 ± 1.4 (5 independent determinations). The protein ratios were similar for comparison with E1β and E2, and for these studies the relative levels of the BCKD catalytic subunits did not change in response to insulin. Additional support for increased steady-state BCKD kinase mRNA was gained by real-time PCR analysis that showed a relative concentration of BCKD kinase mRNA of 0.87 ± 0.11 in cells without insulin and 1.28 ± 0.01 in cells 2 h after insulin administration. This increase of 47% agrees with that determined from Northern blot analysis. The relative concentration remained elevated at 1.16 ± 0.01 in insulin-treated cells for at least 20 h after hormone administration. In sum, the approximately twofold increase in BCKD kinase mRNA and protein closely parallels the reduction in activity state of the BCKD complex after exposure to insulin. Direct measurements of BCKD kinase activity were not possible under the conditions used in these studies.

To assess the contribution of gene transcription to the increase in steady-state BCKD kinase mRNA, cells were treated with the transcriptional inhibitor actinomycin D. In the absence of insulin, actinomycin D treatment resulted in a decrease in BCKD kinase mRNA and protein levels (Fig. 2, *lane 3*). When actinomycin D was added along with insulin, the amount of transcription and translation products was diminished from that observed with insulin alone, but the
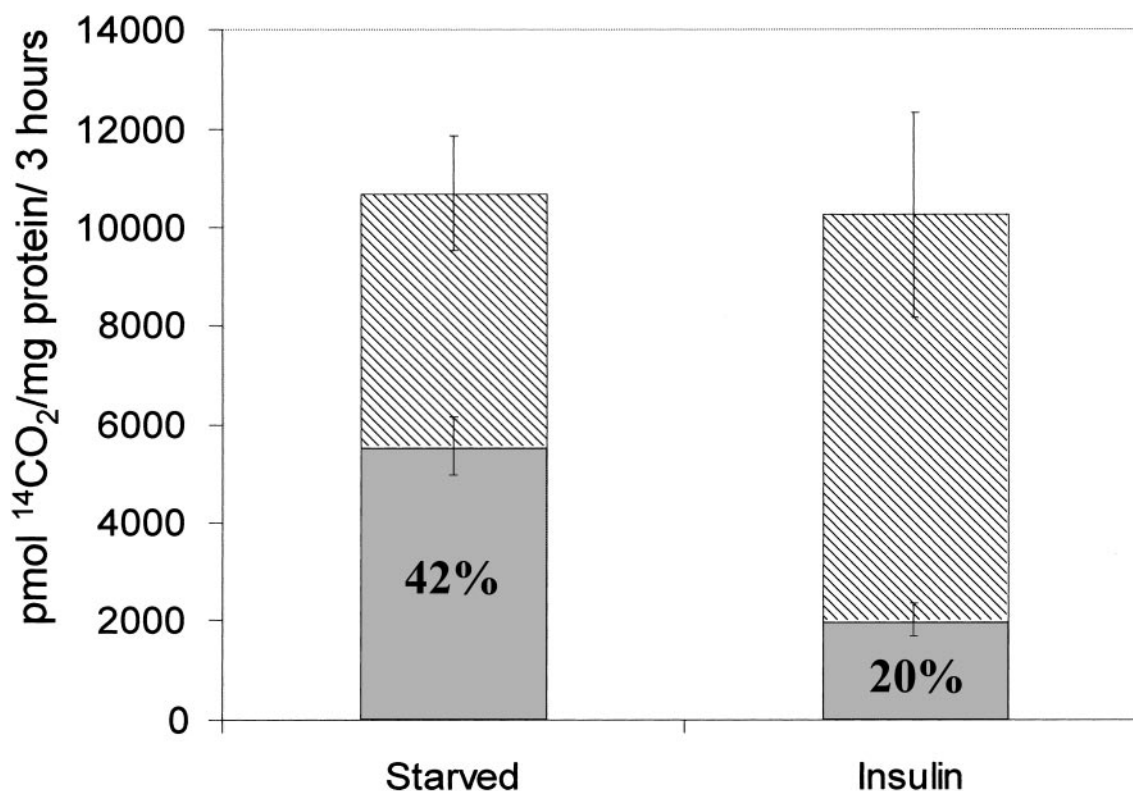
Fig. 1. Branched-chain ketoacid dehydrogenase (BCKD) activity state in serum-starved rat cells treated with 100 nM insulin. Enzyme assay was done 20 h after addition of insulin, and the activity state, indicated as the darker portion in each bar and expressed as %, was calculated as described in METHODS.

ratio of insulin-treated cells vs. control (Fig. 2, *lane 4* vs. *lane 3*) was 2.2 for mRNA and 1.9 for protein. The data support the concept that insulin action does not affect transcription of the BCKD kinase gene. Transcription rate was also analyzed by luciferase reporter assays with the use of three different BCKD kinase promoter/firefly luciferase constructs (described in METHODS) that were cotransfected with *Renilla* luciferase control vectors (pRL-tk, pRL-SV40, and pRL-CMV). All three of the *Renilla* luciferase plasmids demonstrated increased activity in response to insulin treatment, similar to that seen for luciferase, thus hindering the ability to normalize experimental values for transfection efficiency. A previous report by Hwang and Ismail-Beigh (27) used luciferase reporter constructs to study the effect of hyperosmolarity on gene expression in these rat cells. They report no effect on the *Renilla* expression (27). In our study, if luciferase activity in insulin-treated cells was not normalized to the *Renilla* expression, an increased luciferase activity was found in cells incubated in serum-free media supplemented with insulin. Together, the results suggest that insulin does not significantly alter the transcription rate of the BCKD kinase gene, and thus the observed increase in steady-state concentration of BCKD kinase mRNA and protein is more likely due to a change in stability and/or processing of these components. The putative mechanisms for this change were not investigated in this study.

To determine the insulin-signaling pathway(s) involved in the observed changes, cells were pretreated with LY-294002, which inhibits phosphatidylinositol 3-kinase (PI 3-kinase); rapamycin, which inhibits mTOR and activates p70[S6k]; or PD-98059, which inhibits mitogen-activated protein or extracellular signal-regulated kinase (MEK) activation (Fig. 3). All inhibitors act downstream from insulin receptor binding. LY-294002 blocks PI 3-kinase by binding to the p110 subunit and in these studies blocked the increase in BCKD kinase mRNA in response to insulin. BCKD activity state in cells treated with both LY-294002 and insulin was similar to that in serum-starved cells, indicating that blocking the insulin effect on BCKD kinase levels prevented phosphorylation of BCKD (Fig. 3). PI 3-kinase action causes increased phosphorylation of PKB/Akt, a serine/threonine kinase that mediates selected downstream effects of PI 3-kinase. Under these conditions, LY-294002 treatment decreased the phosphorylated form of PKB/Akt (observed by Western blot) in the insulin-treated cells to the level of phosphorylated Akt seen in the serum-starved cells (data not shown).

The activation of p70[S6k] is thought to be downstream of PI 3-kinase and is sensitive to rapamycin (45). Rapamycin treatment caused a partial inhibition of the insulin-stimulated increase of BCKD kinase mRNA. These results suggested that both a rapamycin-sensitive and
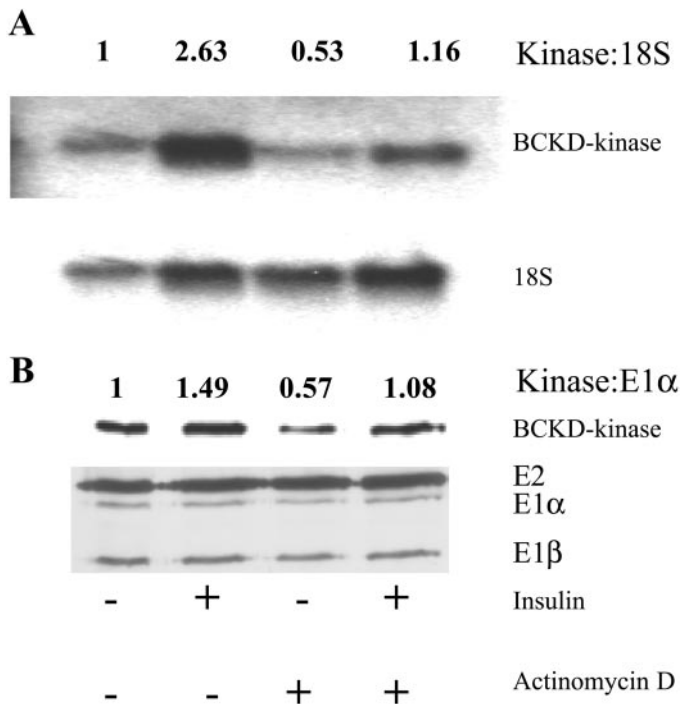
Fig. 2. Effect of insulin administration on expression of BCKD kinase mRNA (*A*) and protein (*B*). Values shown above each lane are based on densitometry measurements and calculated relative to the densitometry values of 18S in each lane. The values reported are normalized to *lane 1* volumes. Steady-state level of BCKD kinase mRNA, assessed by Northern blot, reflected a relative increase of 2.1 ± 0.5 averaged over 4 independent studies (*lane 2* vs. *lane 1*). Steady-state protein was assessed by Western blot with a relative increase of 2.6 ± 1.4 averaged from 5 independent studies ($P = 0.03$ by 1-sided *t*-test; *lane 2* vs. *lane 1*). Note the ability of actinomycin D to block the insulin-stimulated expression of BCKD kinase mRNA and protein (*lanes 3* and *4*). The ratio of *lane 4* to *3* is 2.2 for the Northern blot and 1.9 for the Western blot.

a rapamycin-insensitive pathway may be functioning to produce the observed response to insulin.

Inhibition of MEK (Ras/mitogen-activated protein kinase pathway) by treatment with the inhibitor PD-98059 appeared to have no effect on the insulin-produced increase of BCKD kinase mRNA and the protein expression (data not shown). This could be due to the fact that MEK is already activated, in which case PD-98059 addition would not be inhibitory. The MEK pathway is thought to be active in liver cell survival in serum-depleted medium (39). Data in Fig. 4 demonstrate that the activity state of the BCKD complex was normalized when inhibitors were present along with insulin treatment. Insulin may still have some effect in the presence of the rapamycin (see *lanes 4* and *6* in Fig. 4).

DISCUSSION

In the liver, insulin promotes glycogen synthesis, prevents gluconeogenesis, and is antiproteolytic. The presence of insulin creates an anabolic state where the liver requires BCAA for protein synthesis and therefore BCAA catabolism must be slowed. Our data indicated that insulin upregulated BCKD kinase levels in

cultured rat cells with a concomitant decrease in BCKD activity state. Both BCKD kinase mRNA and protein steady-state concentrations were elevated above control when insulin was present in the culture medium of these rat cells. A major consequence of the induction of BCKD kinase expression by insulin is the preservation of cellular leucine concentration. Because leucine has protein-preserving properties, it appears that leucine acts in concert with insulin to prevent protein degradation through activation of a signaling pathway involving mTOR (50).

Perusal of the nucleic acid sequence in the rat BCKD kinase putative promoter identified four potential insulin response sequence (IRS) sites, with the canonical sequence TATTTTG found at position $-190/-196$, $-248/-254$, $-421/-427$, and $-597/-603$ (26). This element is reported to downregulate gene expression (24). No regions corresponding to the hepatocyte nuclear factor-3β sites reported to be involved in upregulation of genes in response to insulin were apparent in the BCKD kinase promoter (23). Still, the initial observation of increased expression of BCKD kinase could result from increased transcription of this gene. None of the data assessing transcription rates demonstrated an upregulation in response to insulin. Indeed, the observed increase in BCKD kinase expression was best explained by mRNA and/or protein stabilization. Previous studies have shown that insulin can stabilize mRNA, but the mechanism has not been determined (41). We previously have shown that these rat cells responded to deprivation of BCAA by increased translation of BCKD kinase mRNA without changing mRNA levels (17). Rat cells deprived of BCAA for 5 days had a greater amount of BCKD kinase mRNA associated with polyribosomes, indicating that translation of BCKD kinase was increased. The current results suggest that the effect of insulin on BCKD kinase expression is due to posttranscriptional events.

Insulin is known to increase translation through activation of translation initiation and elongation factors (29, 45). The insulin effects seen here were likely dependent on receptor binding, since both the insulin binding and insulin response were lost as the cell doubling increased (data not shown). The signaling pathway depended on PI 3-kinase activation, since LY-294002, a known inhibitor of PI 3-kinase, completely blocked the insulin response of increased in BCKD kinase expression. PI 3-kinase activates PKB/Akt, leading to the phosphorylation and inhibition of GSK-3 or the stimulation of mTOR and p70[S6k], which are sensitive to inhibition by rapamycin (45, 53). By inhibition of PI 3-kinase, mTOR is also blocked, since it is downstream of PI 3-kinase (50). However, the action of insulin does not occur solely through the mTOR pathway, since rapamycin only partially inhibited the induction of BCKD kinase expression. Stimulation of PKB/Akt, p70[S6k], or both via PI 3-kinase activation could lead to increased translation of BCKD kinase (29). Activation of the translation initiation factor eIF2B has been shown to occur through PI 3-kinase activation. Insulin

Fig. 3. Effect of inhibitors of signal transduction on the ability of insulin to increase steady-state levels of BCKD kinase mRNA. *A*: relative concentration of the BCKD kinase mRNA quantified by densitometry and presented as the average ± SE from 2 independent experiments. *B*: autoradiograph of BCKD kinase mRNA and 18S RNA. Insulin was present along with the indicated inhibitor. Concentrations of reagents are described in METHODS. BC, branched-chain.
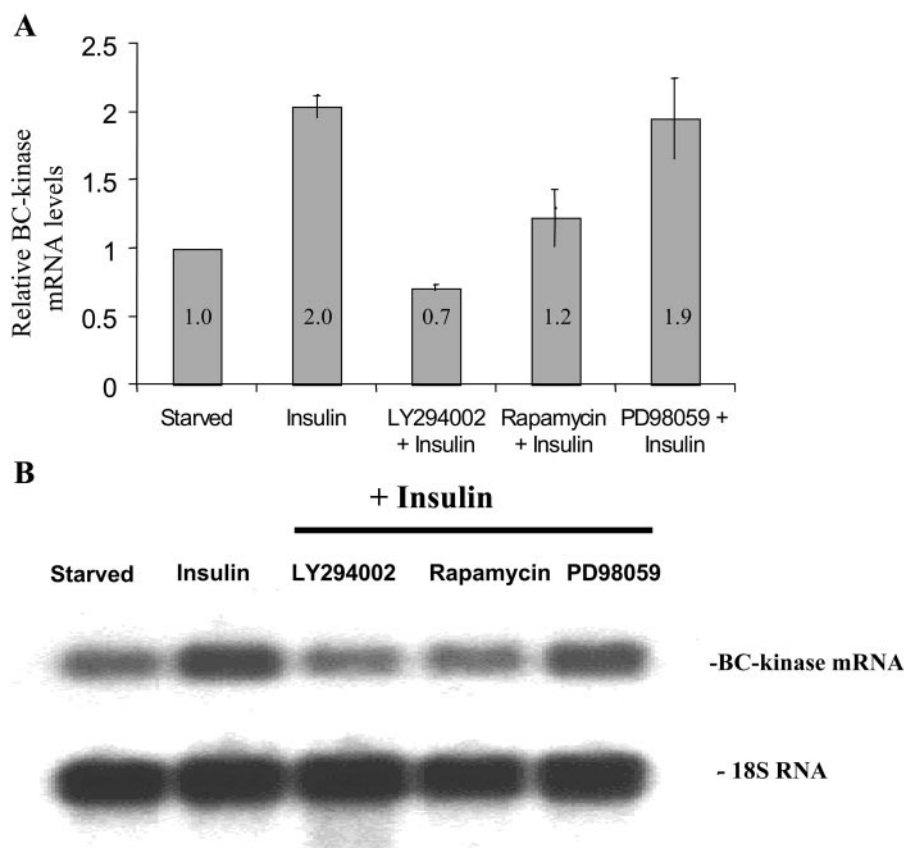
signaling that results in activation of p70[S6k] has been shown to increase translation of mRNA through activation of eukaryotic elongation factor eEF2. Finally, both the PI 3-kinase-activated PKB/Akt pathway and mTOR pathway lead to increased translation through increased phosphorylation of 4E-BP1, which causes release and activation of the translation initiation factor eIF4E. Association of mRNA with ribosomes can act to stabilize the transcript. These rat cells could use a combination of any of these pathways to produce the observed increase in BCKD kinase expression.

Control of BCKD kinase expression is an important regulatory point in BCAA catabolism. In catabolic states such as diabetes, sepsis, cachexia, and aging, where proteolysis is accelerated, it may be possible to boost BCKD kinase levels to decrease the activity state of BCKD and prevent accelerated protein catabolism. We have shown that insulin can increase expression of BCKD kinase in rat cells. Further studies in other tissues will provide a better understanding of the tissue-specific regulation of BCKD kinase expression. By understanding how BCKD kinase expression is regu-

Fig. 4. Activity state of BCKD in the presence of insulin and the various inhibitors. %BCKD in the active state is indicated by the darker portion in each bar.
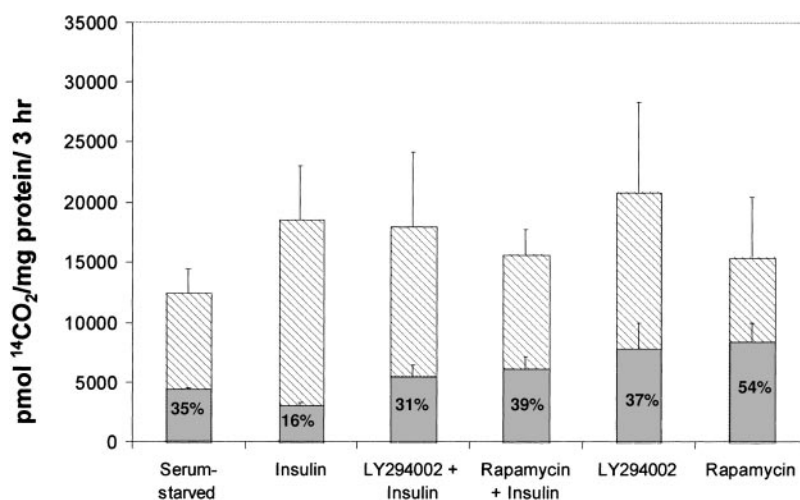
lated in a nondisease state, it may be possible to identify what regulatory factors are disrupted in catabolic diseases and develop therapies to counteract this condition.

REFERENCES

1. **Ævarsson A, Chuang JL, Wynn RM, Turley S, Chuang DT, and Hol WGJ.** Crystal structure of human branched-chain α-ketoacid dehydrogenase and the molecular basis of multienzyme complex deficiency in maple syrup urine disease. *Structure Fold Des* 8: 277–291, 2000.

2. **Ang B, Wade A, Halliday D, and Powell-Tuck J.** Insulin reduces leucine oxidation and improves net leucine retention in parenterally fed humans. *Nutrition* 16: 221–225, 2000.

3. **Antonarakis SE.** Recommendations for a nomenclature system for human gene mutations. *Hum Mutat* 11: 1–3, 1998.

4. **Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, and Struhl K.** *Current Protocols in Molecular Biology*. New York: Greene and Wiley-Interscience, 1989.

5. **Balage M, Sinaud S, Prod'homme M, Dardevet D, Ary TC, Kimball SR, Jefferson LS, and Grizard J.** Amino acids and insulin are both required to regulate assembly of the eIF4E·eIF4G complex in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 281: E565–E574, 2001.

6. **Base W, Barsigian C, Schaeffer A, Shaw E, Martinez J, and Maddrey WC.** Influence of branched-chain amino acids and branched-chain keto acids on protein synthesis in isolated hepatocytes. *Hepatology* 7: 324–329, 1987.

7. **Block KP, Heywood BW, Buse MG, and Harper AE.** Activation of rat liver branched-chain 2-oxoacid dehydrogenase in vivo by glucagon and adrenaline. *Biochem J* 232: 593–597, 1985.

8. **Block KP, Soemitro S, Heywood BW, and Harper AE.** Activation of liver branched-chain α-ketoacid dehydrogenase in rats by excess of dietary amino acids. *J Nutr* 115: 1550–1561, 1985.

9. **Boirie Y, Short KR, Ahlman B, Charlton M, and Nair KS.** Tissue-specific regulation of mitochondrial and cytoplasmic protein synthesis rates by insulin. *Diabetes* 50: 2652–2658, 2001.

10. **Brosnan ME, Lowry A, and Brosnan JT.** Diabetes increases active fraction of branched-chain 2-oxoacid dehydrogenase in rat brain. *Diabetes* 35: 1041–1043, 1986.

11. **Castellino P, Luzi L, Simonson DC, Haymond M, and DeFronzo RA.** Effect of insulin and plasma amino acid concentration on leucine metabolism in man. Role of substrate availability on estimates of whole body protein synthesis. *J Clin Invest* 80: 1784–1793, 1987.

12. **Chua B, Siehl DL, and Morgan HE.** Effect of leucine and metabolites of branched-chain amino acids on protein turnover in heart. *J Biol Chem* 254: 8358–8362, 1979.

13. **Damuni Z, Merryfield ML, Humphreys JS, and Reed LJ.** Purification and properties of branched-chain α-ketoacid dehydrogenase phosphatase from bovine kidney. *Proc Natl Acad Sci USA* 81: 4335–4338, 1984.

14. **Damuni Z and Reed LJ.** Purification and properties of the catalytic subunit of the branched-chain α-ketoacid dehydrogenase phosphatase from bovine kidney mitochondria. *J Biol Chem* 262: 5129–5132, 1987.

15. **Davis TA, Burrin DG, Fiorotto ML, Reeds PJ, and Jahoor F.** Roles of insulin and amino acids in the regulation of protein synthesis in the neonate. *J Nutr* 128: 347S–350S, 1998.

16. **Doering CB, Coursey C, Spangler WE, and Danner DJ.** Murine branched-chain α-ketoacid dehydrogenase kinase: cDNA cloning, tissue distribution, and temporal expression during embryonic development. *Gene* 212: 213–219, 1998.

17. **Doering CB and Danner DJ.** Amino acid deprivation induces translation of branched-chain α-ketoacid dehydrogenase kinase. *Am J Physiol Cell Physiol* 279: C1587–C1594, 2000.

18. **Doering CB, Williams IR, and Danner DJ.** Controlled overexpression of BCKD kinase expression: metabolic engineering applied to BCAA metabolism in a mammalian system. *Metab Eng* 2: 349–356, 2000.

19. **Essén P, Heys SD, Garlick P, and Wernerman J.** The separate and combined effect of leucine and insulin on muscle free amino acids. *Clin Physiol* 14: 513–525, 1994.

20. **Frick GP and Goodman HM.** Insulin regulation of the activity and phosphorylation of branched-chain 2-oxo acid dehydrogenase in adipose tissue. *Biochem J* 258: 229–235, 1989.

21. **Gillim SE, Paxton R, Cook GA, and Harris RA.** Activity state of the branched-chain α-ketoacid dehydrogenase complex in heart, liver, and kidney of normal, fasted, diabetic, and protein-starved rats. *Biochem Biophys Res Commun* 111: 74–81, 1983.

22. **Greiwe JS, Kwon G, McDaniel ML, and Semenkovich CF.** Leucine and insulin activate P70 S6 kinase through different pathways in human skeletal muscle. *Am J Physiol Endocrinol Metab* 281: E466–E471, 2001.

23. **Gronning LM, Cederberg A, Miura N, Enerbäck S, and Taskén K.** Insulin and TNFα induce expression of the forkhead transcription factor gene *Foxc2* in 3T3-L1 adipocytes via PI3K and ERK 1/2-dependent pathways. *Mol Endocrinol* 16: 873–883, 2002.

24. **Guo S, Rena G, Cichy S, He X, Cohen P, and Unterman T.** Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence. *J Biol Chem* 274: 17184–17192, 1999.

25. **Harris RA, Paxton R, Powell SM, Goodwin GW, Kuntz MJ, and Han AC.** Regulation of branched-chain α-ketoacid dehydrogenase complex by covalent modification. *Adv Enzyme Regul* 25: 219–237, 1986.

26. **Huang YS and Chuang DT.** Structural organization of the rat branched-chain 2-oxo acid dehydrogenase kinase gene and partial characterization of the promoter-regulatory region. *Biochem J* 313: 603–609, 1996.

27. **Hwang DY and Ismail-Beigh F.** Stimulation of GLUT-1 glucose transporter expression in response to hyperosmolarity. *Am J Physiol Cell Physiol* 281: C1365–C1372, 2001.

28. **Kasperek GJ, Dohm GL, and Snider RD.** Activation of branched-chain keto acid dehydrogenase by exercise. *Am J Physiol Regul Integr Comp Physiol* 248: R166–R171, 1985.

29. **Kleijn M, Scheper GC, Voorma HO, and Thomas AA.** Regulation of translation initiation factors by signal transduction. *Eur J Biochem* 253: 531–544, 1998.

30. **Lombardo YB, Thamotharan M, Bawani SZ, Paul HS, and Adibi SA.** Posttranslational alterations in protein masses of hepatic branched-chain keto acid dehydrogenase and its associated kinase in diabetes. *Proc Assoc Am Physicians* 110: 40–49, 1998.

31. **Longo N, Langley SD, and Still MJ.** Role of arginine 86 of the insulin receptor in insulin binding and activation of glucose transport. *Biochim Biophys Acta* 1402: 86–94, 1998.

32. **May ME and Buse MG.** Effects of branched-chain amino acids on protein turnover. *Diabetes Metab Rev* 5: 227–245, 1989.

33. **McConnell BB, Burkholder B, and Danner DJ.** Two new mutations in the human E1β subunit of branched-chain α-ketoacid dehydrogenase associated with maple syrup urine disease. *Biochim Biophys Acta* 1361: 263–271, 1997.

34. **McKenzie S, Phillips SM, Carter SL, Lowther S, Gibala MJ, and Tarnopolsky MA.** Endurance exercise training attenuates leucine oxidation and BCOAD activation during exercise in humans. *Am J Physiol Endocrinol Metab* 278: E580–E587, 2000.

35. **Meek SE, Persson M, Ford GC, and Nair KS.** Differential regulation of amino acid exchange and protein dynamics across splanchnic and skeletal muscle beds by insulin in healthy human subjects. *Diabetes* 47: 1824–1835, 1998.

36. **Miller RH, Eisenstein RS, and Harper AE.** Effects of dietary protein intake on branched-chain keto acid dehydrogenase activity of the rat. Immunochemical analysis of the enzyme complex. *J Biol Chem* 263: 3454–3461, 1988.

37. **Miotto G, Venerando R, Khurana KK, Siliprandi N, and Mortimore GE.** Control of hepatic proteolysis by leucine and isovaleryl-L-carnitine through a common locus. Evidence for a

possible mechanism of recognition at the plasma membrane. *J Biol Chem* 267: 22066–22072, 1992.

38. **Mitch WE and Clark AS.** Specificity of the effect of leucine and its metabolites on protein degradation in skeletal muscle. *Biochem J* 222: 579–586, 1984.

39. **Mitsui H, Takuwa N, Maruyama T, Maekawa H, Hirayama M, Sawatari T, Hashimoto N, Takuwa Y, and Kimura S.** The MEK1-ERK MAP kinase pathway and the PI 3-kinase-AKT pathway independently mediate anti-apoptotic signals in HEPG2 liver cancer cells. *Int J Cancer* 92: 55–62, 2001.

40. **Nair KS, Ford GC, Ekberg K, Fernqvist-Forbes E, and Wahren J.** Protein dynamics in whole body and in splanchnic and leg tissues in type I diabetic patients. *J Clin Invest* 95: 2926–2937, 1995.

41. **O'Brien RM and Granner DK.** Regulation of gene expression by insulin. *Physiol Rev* 76: 1109–1161, 1996.

42. **Patston PA, Espinal J, and Randle PJ.** Effects of diet and of alloxan diabetes on the activity of branched-chain 2-oxoacid dehydrogenase complex and of activator protein in rat tissues. *Biochem J* 222: 711–719, 1984.

43. **Popov KM, Zhao Y, Shimomura Y, Jaskiewicz J, Kedishvili NY, Irwin J, Goodwin GW, and Harris RA.** Dietary control and tissue specific expression of branched-chain α-ketoacid dehydrogenase kinase. *Arch Biochem Biophys* 316: 148–154, 1995.

44. **Popov KM, Zhao Y, Shimomura Y, Kuntz MJ, and Harris RA.** Branched-chain α-ketoacid dehydrogenase kinase. Molecular cloning, expression and sequence similarity with histidine protein kinase.*J Biol Chem* 267: 13127–13130, 1992.

45. **Proud CG and Denton RM.** Molecular mechanisms for the control of translation by insulin. *Biochem J* 328: 329–341, 1997.

46. **Quintanilla RA, Porras OH, Castro J, and Barros LF.** Cytosolic [Ca^{2+}] modulates basal GLUT1 activity and plays a permissive role in its activation by metabolic stress and insulin in rat epithelial cells. *Cell Calcium* 28: 97–106, 2000.

47. **Shah BH, Olivares-Reyes JA, Yesilkaya A, and Catt KJ.** Independence of angiotensin II-induced MAP kinase activation from angiotensin type 1 receptor internalization in clone 9 hepatocytes. *Mol Endocrinol* 16: 610–620, 2002.

48. **Shetty M, Kuruvilla AK, Ismail-Beigi F, and Loeb JN.** Stimulation of glucose transport in clone 9 cells by insulin and thyroid hormone: role of GLUT-1 activation. *Biochim Biophys Acta* 1314: 140–146, 1996.

49. **Shimomura Y, Fujii H, Suzuki M, Murakami T, Fujitsuka N, and Nakai N.** Branched-chain α-ketoacid dehydrogenase complex in rat skeletal muscle: regulation of the activity and gene expression by nutrition and physical exercise. *J Nutr* 125: 1762S–1765S, 1995.

50. **Van Sluijters DA, Dubbelhuis PF, Blommaart EFC, and Meijer AJ.** Amino-acid-dependent signal transduction. *Biochem J* 351: 545–550, 2000.

51. **Wagenmakers AJM, Brookes JH, Coakley JH, Reilly T, and Edwards RHT.** Exercise-induced activation of the branched-chain 2-oxo acid dehydrogenase in human muscle. *Eur J Appl Physiol* 59: 159–167, 1989.

52. **Wagenmakers AJM, Schepens JTG, Veldhuizen JAM, and Veerkamp JH.** The activity state of the branched-chain 2-oxoacid dehydrogenase complex in rat tissues. *Biochem J* 220: 273–281, 1984.

53. **Wagle A, Jivraj S, Garlock GL, and Stapleton SR.** Insulin regulation of glucose-6-phosphate dehydrogenase gene expression is rapamycin-sensitive and requires phosphatidylinositol 3-kinase. *J Biol Chem* 273: 14968–14974, 1998.

54. **Yeaman SJ.** The 2-oxo acid dehydrogenase complexes: recent advances. *Biochem J* 257: 625–632, 1989.