Effects of insulin on the regulation of branched-chain α-keto acid dehydrogenase E1α subunit gene expression

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Alterations in dietary intake, especially of protein, may produce changes in the hepatic levels of the branched-chain α-keto acid dehydrogenase (BCKAD) complex. The possible role of insulin in the regulation of these observed changes in hepatic capacity for BCKAD expression was therefore examined. Steady-state RNA levels encoding three of the subunits, E1α, E1β and E2, increased by 2–4-fold in the livers of mice starved for 3 days, a known hypoinsulinaemic state. In contrast, the levels of E1β and E2, but not E1α, RNA were decreased when mice were fed 0% protein diets compared with the levels observed in mice fed standard (23%) or higher protein isocaloric diets. BCKAD subunit protein levels under these conditions changed co-ordinately even though the changes in RNA were not co-ordinate. The effects of hormonal changes that might be associated with these dietary changes were examined, using the rodent hepatoma cell line H4IIEC3. In these cells, the levels of E1α protein and mRNA were significantly depressed in the presence of insulin. In contrast, the levels of E1β and E2 RNAs were not decreased by insulin. The half-lives of the E1α and E2 RNAs were determined to be quite long, from 13 to 18 h, with insulin having no dramatic overall effect on the half-lives determined over 24 h. Therefore, it is likely that insulin directly affects the transcription of the E1α gene rather than RNA stability in exerting its negative regulatory effect. This effect is specific to the E1α subunit. The differences in BCKAD subunit RNA levels observed under various nutritional and developmental conditions may therefore be the result of the differential effects of insulin and other hormones on the multiple regulatory mechanisms modulating BCKAD subunit expression.

INTRODUCTION

Branched-chain α-keto acid dehydrogenase (BCKAD, EC 1.2.4.4) is the mitochondrial multimeric enzymic complex which catalyses the oxidative decarboxylation of the branched-chain α-keto acids derived from the branched-chain amino acids (BCAAs) leucine, isoleucine and valine. The enzymic activities comprising the BCKAD complex include α-keto acid decarboxylase (E1, EC 1.2.4.4), dihydrolipoamide acyltransferase (E2, no EC number) and dihydrolipoamide reductase (E3, EC 1.8.1.4). The E1 decarboxylase is composed of two different polypeptides, E1α and E1β. The BCKAD complex appears to be composed of 24 E1α and E1β proteins (associated as 12 $\alpha_2\beta_2$ subunits), 24 E2 subunits, six E3 dimers, an undetermined number of BCKAD kinase and phosphatase molecules, and several bound cofactors (reviewed in [1,2]).

Enzyme activity appears to be highly regulated through a phosphorylation (inactivation)–dephosphorylation (activation) mechanism that involves associated BCKAD kinase (EC 2.7.1.115) and phosphatase (EC 3.1.3.52) proteins acting upon the E1α subunit [3–5]. Nutritional state, exercise, and endocrine factors are among the many influences which have been noted to affect the degree of phosphorylation and therefore the relative activity state of the enzymic complexes present at any given time [6–15]. BCKAD activity is thought to be present in all mammalian tissues but at varying levels, with the liver demonstrating the highest activity per organ [6,14]. Because the liver contains the majority of the total body BCKAD capacity, it is felt to have a principal role in directing the overall balance between catabolism of the BCAAs versus their utilization for protein synthesis as essential amino acids [14].

The response of hepatic BCKAD activity to changes in dietary protein has been addressed by several laboratories. Measurements of enzymic activity have provided inconsistent results on the extent that changes in total BCKAD content, as opposed to changes in activity state due to phosphorylation, contribute to alterations in liver BCKAD activity in response to dietary protein [8,13–17]. More recently, examination of the steady-state levels of RNAs encoding BCKAD subunits (E1α, E1β, and E2) in rodent tissues obtained from animals fed diets with various protein contents or at different post-natal developmental ages suggested that regulation of BCKAD subunit gene expression was involved in these responses [18–20]. In this report, we present our studies focusing on changes in hepatic capacity for BCKAD expression in response to diet. Our results suggest that insulin affects the expression of BCKAD E1α and this subunit-specific response may be the basis for the non-co-ordinated changes in BCKAD RNAs observed under certain dietary conditions [19,20].

EXPERIMENTAL

Animals

C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, ME, U.S.A. The diets used were obtained from Purina Test Diets (Richmond, IN, U.S.A.) and included standard Laboratory Rodent Diet (#5001) containing 23.0% protein, 4.5% fat and 49.0% carbohydrate; Protein Free Purified Diet (#5765C) containing 0% protein, 10.0% fat and 81.70% carbohydrate; 6% Protein Purified Diet (5767C-G) containing 6.0% protein, 10.3% fat and 75.13% carbohydrate; 23% Protein

Abbreviations used: BCKAD, branched-chain α-keto acid dehydrogenase complex; BCAAs, branched-chain amino acids; E1, α-keto acid decarboxylase; E2, dihyrolipoamide acyltransferase; E3, dihydrolipoamide dehydrogenase; AS, argininosuccinate synthetase; AL, argininosuccinate lyase.

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Purified Diet (#5776C-I) containing 23.0 % protein, 10.0 % fat and 56.08 % carbohydrate; 50 % Protein Purified Diet (#5786C-3) containing 50.5 % protein, 13.5 % fat and 23.35 % carbohydrate; and 60 % Protein/Low Carbohydrate Diet (#5783C-E) containing 60.0 % protein, 10 % fat and 1.9 % carbohydrate. The Protein Purified Diets are isocaloric, providing 4.1 kcal/g of digestible energy, while standard diet and 60 % Protein/Low Carbohydrate diets contain 3.3–3.4 kcal/g of digestible energy. All of the diets contained similar mineral and fibre contents. The mice were fed these diets and water on an *ad libitum* basis. For the starvation experiments, the food was removed but water *ad libitum* was maintained. Experimental groups, containing at least four mice per group, were age, sex and litter-matched. All experiments reported were repeated at least twice with different groups of mice. Mice were weighed at the time of arrival, then randomized following a period of acclimatization on standard laboratory chow and weighed again at the start and completion of each experiment. Only female mice were used because their weight gain at this age (6.5–8.5 weeks) is minimal compared with male mice and standard weight gain curves were available from the Jackson Laboratories. Results are shown for those experimental groups of mice who consumed equivalent volumes of food and as a group demonstrated similar weight changes during the experimental periods. In general, following 3 day starvation, C57BL/6J female mice would lose 25–28 % body weight ($n = 16$ in four experiments), compared with no significant weight changes ($\pm$3–4 %) on any of the diets described over the same 3 day period. No ketonuria was observed with any of the diets except following 3 day starvation.

Isolation and blot hybridization analysis of steady-state RNA

Total cellular RNA was isolated from murine tissues by the guanidinium isothiocyanate–phenol extraction method, separated by electrophoresis in 1 % agarose–formaldehyde gels, transferred to nylon membranes (MagnaGraph, MSI, Westboro, MA, U.S.A.) and hybridized with random-primed ^{32}P-labelled cDNA as previously described [20,21]. For some experiments, hybridization analyses were performed using Rapid-Hyb Buffer (Amersham Life Science) with no differences in the results obtained. Ethidium bromide staining of 28 S and 18 S ribosomal RNA confirmed equivalent loading in all lanes. Rehybridization of all blots with several cDNA probes (see below) was performed to ensure relative equality of different RNA preparations. All individual RNA preparations were retested several times to confirm any results presented.

The relative amounts of the mRNAs were determined by densitometric analysis (Bio-Rad Model 620 Video Densitometer) of the autoradiograms of the hybridized blots. Results are reported as the mean $\pm$ S.D. of integrated peak areas representing relative absorbance values obtained from the densitometric analysis (2D Analyst, Bio-Rad). Only values obtained from samples analysed in the same agarose gel and autoradiogram were directly compared. Statistical analysis was performed using Student's *t* test, as previously described [20]. To estimate the half-lives of the BCKAD transcripts, linear regression analysis of the densitometric values obtained from the autoradiograms was used, comparing relative RNA accumulation to length of exposure time to actinomycin D.

Cell culture

Rat hepatoma H4IIEC3 cells were obtained from the American Type Culture Collection and maintained in RPMI (GIBCO) containing 10 % (v/v) fetal-calf serum (BioWhittaker) at 37 °C in a humidified 5 % CO_2/95 % air atmosphere. Specific experimental conditions are indicated in the Figure legends but were set up in an identical manner by plating 1.0×10^6 cells in serum-containing medium for 24 h, feeding the cells with serum-free medium for 24 h, and then adding either insulin (0.1 μM) and/or dexamethasone (1 μM) in serum-free media for the indicated time interval prior to harvesting for BCKAD RNA or protein. When leucine or glucose concentrations were varied, glucose-free RPMI (containing 0.38 mM leucine) was used for the preincubation and glucose (1 mM or 11 mM) and, if indicated, leucine (10 mM) were added to fresh glucose-free RPMI used for the indicated time period (usually 24 h). Actinomycin D (2 μg/ml) was added to serum-free RPMI with or without insulin (0.1 μM) as indicated. Preliminary studies indicated this to be the highest dose associated with > 90 % inhibition of transcription that permitted cell survival in serum-free medium for the interval period required for the experiment ($\geqslant$ 24 h) (results not shown).

Western blot analysis

Cellular protein was extracted for BCKAD subunit immunoblot analysis as described [19]. Cells were lysed directly on the plates using 2 % (w/v) SDS, 10 mM Tris/HCl, pH 6.8, 10 % (w/v) glycerol, 0.1 mM EDTA and 5 % (v/v) 2-mercaptoethanol. The suspension was boiled for 5 min, sonicated on ice twice for 15 s each, and centrifuged at 10000 ***g*** for 5 min. Equivalent amounts of protein were boiled and resolved by electrophoresis through 8.5 % acrylamide/0.2 % bisacrylamide/0.1 % SDS as described [22]. Portions of the gel, or parallel gels containing identical samples, were divided for protein visualization with Coomassie Blue stain or electroblot transfer to nitrocellulose filters (Nitrobind, MSI Inc., Westboro, MA, U.S.A.). The entire filter was stained with Ponceau Red for localization of molecular-mass markers and then washed with PBS. The filter was blocked with 4 % BSA/0.2 % Triton X-100/150 mM NaCl, 10 mM Na_3PO_4, pH 7.5, 1 mM EGTA, 0.05 % sodium azide for 4 h and then incubated with a mixture of polyclonal antisera specific against the BCKAD E1 and E2 subunits overnight at 4 °C. The filters were then extensively washed at room temperature with 0.5 % Triton X-100, 50 mM triethanolamine, pH 7.4, 100 mM NaCl, 2 mM EDTA and 0.1 % SDS. Washed filters were incubated with 1 μCi of ^{125}I-labelled *Staphylococcus aureus* Protein A (ICN, Irvine, CA, U.S.A.) blocking buffer for 1 h, vigorously washed in the above wash buffer, and then exposed to X-ray film (X-Omat AR film, Eastman-Kodak, Rochester, NY, U.S.A.) at -85 °C.

DNA probes

The following ^{32}P-labelled probes were used for Northern blot hybridization experiments: 1.4 kb *Eco*R1 fragment from murine BCKAD E1β cDNA (Genbank accession no. L16992), 1.7 kb *Eco*R1 fragment from murine BCKAD E1α cDNA (see below, Genbank accession no. L47335), and 1.4 kb *Eco*R1–*Cla*1 fragment from murine BCKAD E2 cDNA (Genbank accession no. L42996) containing sequences encoding only the mature preprotein [23,24]; CHOB [25] (kindly provided by Dr. R. Rohan); argininosuccinate synthetase (AS) and lyase (AL) cDNAs [26,27] (kindly provided by Dr. Marian Jackson). The 0.6 kb *Eco*R1–*Bam*H1 fragment of CHOB detects a single RNA species in rodents and is considered to be the mRNA for ribosomal protein S2 [28]. This hamster sequence is 95 % identical to the rat cDNA sequence for ribosomal protein S2 (Genbank accession no. L22552; [28a]).

Cloned murine E1α cDNAs were isolated from both liver and skeletal muscle cDNA libraries [cloned into λZAP vectors (Stratagene) which were the generous gift of the laboratory of

Dr. C. T. Caskey, Baylor College of Medicine, Houston, TX, U.S.A.] using published rat E1α cDNA (the generous gift of Dr. R. Harris, Indiana University School of Medicine, Indianapolis, IN, U.S.A.) [29]. The largest cDNAs isolated were sequenced by preparing a series of cloned overlapping deletion sequences using variably timed exonuclease III/mung bean nuclease digestion and subsequently determining the nucleotide sequence using the dsDNA Cycle Sequencing System (BRL Life Technologies, Inc.). Portions of the clones were also sequenced using cycle sequencing on the Applied Biosystems Automated Sequencer (Model 373A). Sequence comparisons with human and rat E1α were performed using the GCG Wisconsin Package sequence analysis software [29–31].

Materials

The majority of chemical reagents were obtained from Sigma (St. Louis, MO, U.S.A.). Random-priming labelling was performed using the Megaprime DNA labelling system (Amersham Life Sciences). Guanidinium isothiocyanate was obtained from Fluka Chemical Corporation. [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from NEN Research Products (Boston, MA, U.S.A.).

RESULTS

Murine cDNA sequence for BCKAD E1α

The complete sequence of the murine E1α cDNA was determined from cloned cDNA isolated from a murine skeletal cDNA library whose size was consistent with the estimated size of 1.7–1.8 kb observed for hybridized RNA from several murine and rat tissues (see Figure 1) [18–20,23]. The cloned E1α cDNA sequence consists of 1681 bp, with a translation initiation codon, ATG, starting at position 18, a likely polyadenylation signal, AAGTAAA, at bp 1634–1640, and a 12 bp poly-A tail. It demonstrates 94.5 % amino acid and 87.9 % nucleotide identity with the reported human E1α cDNA sequence for the mature protein region (1206 nucleotides, 401 amino acids), but only 68.3 % amino acid and 74.2 % nucleotide identity in the 5′ preprotein region (first 41 amino acids encoded by 123 nucleotides) [30,31]. In contrast, it is much more similar to the cDNA sequence reported for the related rat species, with 89.5 % nucleotide identity in the preprotein region and 96.1 % nucleotide identity in the mature protein coding region [29]. In all three species, hydropathy plots for the preprotein region were similar despite the predicted amino acid non identity (results not shown).

Dietary influences on hepatic expression of BCKAD subunit RNAs and peptides

The levels of BCKAD mRNAs for E1α, E1β and E2 in murine liver tissues from the inbred strain C57BL/6J subjected to dietary changes were determined. Since previous studies had indicated changes associated with dietary protein when isocaloric diets were used, we examined the effect of dietary starvation compared with the effect of isocaloric 0 % protein intake (Figure 2) [20]. In agreement with previous studies, a 2–4-fold decrease in the steady-state levels of BCKAD E1β and E2, but not E1α, RNAs was observed when mice were fed a diet restricted for protein (0 %) but as adequate in calories as the higher-protein diets (23 %) for 3 days. In contrast, following a 3 day starvation period where no calories were ingested (but with water *ad*

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  1 CCG GCG ATA GTG ATG TCT GCG GCC AAG ATC TGG AGG CCG AGC CGT GGC CTG CGC CAG GCT GCT CTT CTC CTG TTG GGA CGA TCT GGG GTT CGG GGC   96
                  MET Ser Ala Ala Lys Ile Trp Arg Pro Ser Arg Gly Leu Arg Gln Ala Ala Leu Leu Leu Gly Arg Ser Gly Val Arg Gly
                 -41
 97 TTG GCT AGA TCT CAC CCC AGC AGG CAG CAG CAA CAG CAG TTC CCA TCC CTG GAC GAC AAG CCC CAG TTC CCA GGG GCC TCT GCA GAG TTT GTA GAC  192
    Leu Ala Arg Ser His Pro Ser Arg Gln Gln Gln Gln Phe Pro Ser Leu Asp Asp Lys Pro Gln Phe Pro Gly Ala Ser Ala Glu Phe Val Asp
                                                    +1
193 AAG CTT GAG TTC ATC CAG CCC AAT GTC ATC TCC GGC ATC CCC ATC TAC CGT GTC ATG GAC CGC CAG GGC CAG ATC ATC AAC CCC AGT GAA GAT CCC  288
    Lys Leu Glu Phe Ile Gln Pro Asn Val Ile Ser Gly Ile Pro Ile Tyr Arg Val MET Asp Arg Gln Gly Gln Ile Ile Asn Pro Ser Glu Asp Pro

289 CAC CTG CCC CAG GAG GAG GTG CTG AAG TTC TAC CGG AGC ATG ACG CTG CTC AAC ACC ATG GAC CGC ATT CTC TAT GAG TCC CAG CGG GAG GGC CGG  384
    His Leu Pro Gln Glu Glu Val Leu Lys Phe Tyr Arg Ser MET Thr Leu Leu Asn Thr MET Asp Arg Ile Leu Tyr Glu Ser Gln Arg Glu Gly Arg

385 ATC TCC TTC TAC ATG ACC AAC TAT GGC GAG GAG GGG ACA CAT GTG GGC AGT GCC GCT GCC TTG GAG CGC ACA GAC CTG GTG TTT GGC CAG TAC CGG  480
    Ile Ser Phe Tyr MET Thr Asn Tyr Gly Glu Glu Gly Thr His Val Gly Ser Ala Ala Ala Leu Glu Arg Thr Asp Leu Val Phe Gly Gln Tyr Arg

481 GAG GCA GGT GTG CTC ATG TAC CGG GAC TAC CCG CTG GAG CTG TTC ATG TCC CAG TGC TAC GGC AAC GTG AAT GAC CCA GGC AAG GGA CGC CAG ATG  576
    Glu Ala Gly Val Leu MET Tyr Arg Asp Tyr Pro Leu Glu Leu Phe MET Ser Gln Cys Tyr Gly Asn Val Asn Asp Pro Gly Lys Gly Arg Gln MET

577 CCT GTT CAC TAC GGT TGC AAG GAA CGC CAC TTC GTC ACC ATT TCT TCT CCA CTG GCC ACG CAG ATC CCT CAG GCG GTA GGG GCA GCC TAT GCT GCC  672
    Pro Val His Tyr Gly Cys Lys Glu Arg His Phe Val Thr Ile Ser Ser Pro Leu Ala Thr Gln Ile Pro Gln Ala Val Gly Ala Ala Tyr Ala Ala

673 AAG CGG GCC AAT GCC AAC CGG ATT GTG ATC TGT TAC TTT GGC GAG GGG GCA GCC AGT GAA GGG GAT GCC CAC GCC GGT TTC AAT TTC GCT GCC ACC  768
    Lys Arg Ala Asn Ala Asn Arg Ile Val Ile Cys Tyr Phe Gly Glu Gly Ala Ala Ser Glu Gly Asp Ala His Ala Gly Phe Asn Phe Ala Ala Thr

769 CTG GAG TGT CCC ATC ATC TTC TTC TGC CGG AAC AAT GGC TAT GCC ATC TCC ACA CCA ACC TCT GAG CAG TAC CGT GGG GAT GGC ATA GCG GCT CGG  864
    Leu Glu Cys Pro Ile Ile Phe Phe Cys Arg Asn Asn Gly Tyr Ala Ile Ser Thr Pro Thr Ser Glu Gln Tyr Arg Gly Asp Gly Ile Ala Ala Arg

865 GGC CCT GGG TAC GGT ATC AAG TCA ATC CGT GTG GAC GGC AAC GAT GTG TTT GCT GTG TAC AAT GCC ACT AAG GAG GCC CGA CGG CGG GCT GTG GCT  960
    Gly Pro Gly Tyr Gly Ile Lys Ser Ile Arg Val Asp Gly Asn Asp Val Phe Ala Val Tyr Asn Ala Thr Lys Glu Ala Arg Arg Arg Ala Val Ala

961 GAG AAC CAG CCC TTC CTC ATT GAG GCC ATG ACC TAC AGG ATT GGC CAC CAC AGC ACC AGT GAC GAC AGC TCA GCG TAC CGC TCG GTG GAC GAG GTC 1056
    Glu Asn Gln Pro Phe Leu Ile Glu Ala MET Thr Tyr Arg Ile Gly His His Ser Thr Ser Asp Asp Ser Ser Ala Tyr Arg Ser Val Asp Glu Val

1057 AAT TAC TGG GAC AAG CAG GAC CAC CCA ATC TCG AGG CTG AGG CAG TAC CTG CTG AAC CAG GGT TGG TGG GAT GAG GAA CAG GAG AAG GCC TGG CGG 1152
     Asn Tyr Trp Asp Lys Gln Asp His Pro Ile Ser Arg Leu Arg Gln Tyr Leu Leu Asn Gln Gly Trp Trp Asp Glu Glu Gln Glu Lys Ala Trp Arg

1153 AAG CAG TCA CGA AAG AAG GTC ATG GAA GCC TTT GAG CAG GCT GAG CGG AAG CTA AAG CCG AAC CCA AGC CTC CTC TTC TCC GAT GTG TAC CAG GAG 1248
     Lys Gln Ser Arg Lys Lys Val MET Glu Ala Phe Glu Gln Ala Glu Arg Lys Leu Lys Pro Asn Pro Ser Leu Leu Phe Ser Asp Val Tyr Gln Glu

1249 ATG CCC GCC CAG CTC CGC AGG CAG CAG GAG TCG CTG GCA CGG CAC CTG CAG ACT TAC GGG GAG CAC TAC CCC CTG GAC CAC TTT GAA AAG TGA GGC 1344
     MET Pro Ala Gln Leu Arg Arg Gln Gln Glu Ser Leu Ala Arg His Leu Gln Thr Tyr Gly Glu His Tyr Pro Leu Asp His Phe Glu Lys  *
                                                                                                          +401
1345 CCT AGC CAG CAC AAG GGG GTC CTG CTC TGA GTG GAT GCT GCC CCC TGT GCT AGT CAG CTG TGC GAA CAT GCT GAG CAG CCA GGG TGG CAT TCC AGG 1440
1441 GTG ACT TTT GCT CCT GTT CCT GCT CCC CCA GGC TAC TTC ACT GGG GCA CCA TGT TTG CAG CCA TTG CTG CGG CTA CTT CAC TCC CTT CAC CTG TTA 1536
1537 CAG TAC CTT TTA GGG GTG GGA AGG GAC CAG GGC CAG GGA AGA CCC TCT GGG CAG GGT GGC CCT GGG CTT GCC CAG AGA GAT AGC TGA TTG GCA GAG 1632
1633 TAA GTA AAC CAC AGG TCT GCC TTT GAA AAA AAA AAA AGG AAT TC 1676
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Figure 1 Sequence of murine BCKAD E1α cDNA

The cDNA sequence is shown with the deduced encoding peptide sequence below. The location of the initiation codon for methionine (AUG), the first, and the last amino acids of the mature peptide are numbered below the indicated codon sequence. The termination codon is marked with an asterisk (*). The nucleotide position is indicated on both sides of the sequence.

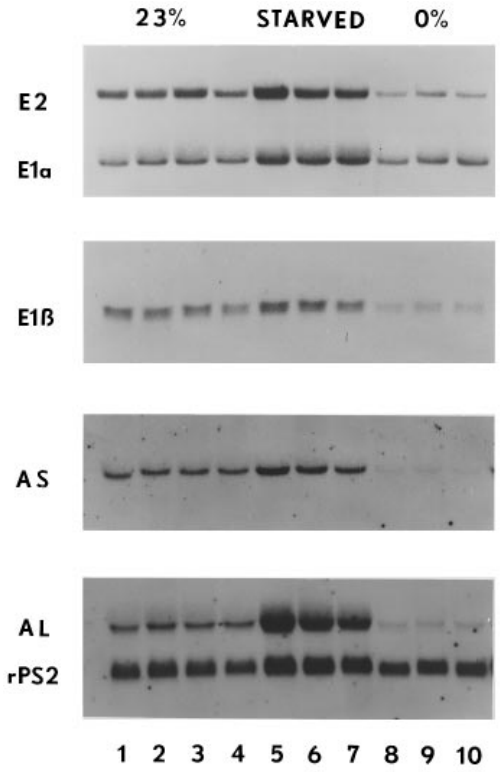

Figure 2 Effect of dietary changes on levels of BCKAD subunit RNAs in murine liver

Samples (20 μg) of RNA from individual C57BL/6J mice ($n = 4$ for 23% and $n = 3$ for 0% protein diets, $n = 3$ for starved animals) were hybridized with the murine BCKAD cDNA probes indicated. The protein diets given to the mice prior to organ removal are indicated (0%, 23% protein) as are the mice starved for 72 h. All samples were rehybridized with cDNA probes to detect ribosomal protein S2 (rPS2) and actin RNAs (not shown in this specific Figure but see Figure 3) to determine the comparable qualities of the RNA between lanes.

Table 1 Changes in BCKAD subunit RNAs following 3 day starvation in mice

Cloned cDNA sequences for murine BCKAD subunits E1α, E1β, and E2, as well as actin, were hybridized to liver RNA obtained from mice ($n = 4$) placed on a 23% protein isocaloric diet for 7 days and then continued on a 23% isocaloric diet or starved for 3 days, as described in the Experimental section. The resulting autoradiograms were analysed by densitometry. Mean densitometric absorbance units (relative to background) $\pm$ S.D. for each BCKAD probe are indicated. Fold increase represents the ratio between the mean values obtained following the switch from 23% protein to 3 day starvation. *Significant changes by Student *t*-test at the 95% confidence level.

	Densitometric absorption units		
	23% Protein	Starvation	Fold increase
E1α	5.6 $\pm$ 0.9	13.3 $\pm$ 1.21	2.4*
E1β	6.6 $\pm$ 2.8	24.8 $\pm$ 4.3	3.8*
E2	3.3 $\pm$ 0.7	13.8 $\pm$ 0.6	4.2*
Actin	7.2 $\pm$ 0.9	8.10 $\pm$ 0.7	1.1

libitum), the levels of all three BCKAD RNAs increased in the liver (see Figure 2 and Table 1). The levels of RNAs encoding two urea cycle enzymes (AS and AL) were higher with the higher-protein-content diet (23%) as well as following starvation,

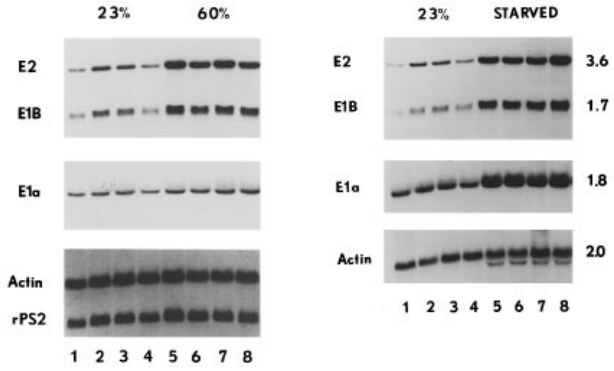

Figure 3 Effects of 60% protein diet versus starvation on hepatic BCKAD subunit RNA levels in mice previously fed a 23% protein diet

Northern blot hybridization of 20 μg of total liver RNA obtained from 7-week-old C57BL/6J mice ($n = 4$) placed on a 23% protein diet for 7 days and then switched to a 60% protein isocaloric diet or starved for 72 h. Cloned cDNA sequences for murine BCKAD subunits were hybridized first, and subsequently, several probes including ribosomal protein S2 (rP-S2), actin and AS and AL were used for rehybridization. The sizes of the RNA species, in kilobase pairs, are given on the right. The unlabelled 1.8–1.9 kb band below the actin RNA in the starved samples is due to residual hybridization with a prior AL cDNA probe which did not completely wash off.

which is consistent with previous reports [32]. Starvation resulted in no significant changes in the steady-state levels of actin RNA but a slight increase in the levels of ribosomal protein S2 RNA. While on a 0% protein diet for 3 days, no significant changes in weight were observed ($\pm$4%). In contrast, following 3 day starvation, weight losses of 20–28% were routinely observed. It would appear that protein catabolism induced by diets containing 0% protein but adequate fat and carbohydrate affected BCKAD gene expression differently than starvation-induced catabolism, in which protein breakdown is required to meet ongoing energy requirements as well as the needs of protein synthesis. Densitometric analysis of several hybridized RNA blots suggested that the increases in BCKAD E1β and E2 were of the order of 3–5-fold, while the increase in E1α was slightly less, about 2-fold (Table 1).

In contrast to the non-co-ordinated changes in subunit RNAs associated with alterations in the protein composition of isocaloric diets, caloric deprivation through starvation resulted in a co-ordinated increase in all three RNAs. The effects of another dietary extreme, 60% protein/2% carbohydrate (from the required vitamin supplement), which contains adequate calories (3.38 kcal/g versus 3.29 kcal/g for standard laboratory 23% protein diet), was also compared with starvation (Figure 3). Mice were fed a 23% Protein Purified Diet for 7 days and subsequently starved for 3 days. In parallel, mice were changed to this 60% protein diet for 3 days. No significant weight change was observed with this diet, and therefore, it was expected that this diet would increase protein utilization to meet energy requirements, and capacity for BCKAD expression would increase. As shown in Figure 3, similar to our previous results, following starvation all three BCKAD RNAs increased. In contrast, following the high-protein/low-carbohydrate diet, only E1β and E2 significantly increased. These studies suggested that the two different dietary extremes that still provided adequate calories for growth produced similar patterns of non-co-ordinate change in BCKAD RNA levels. BCKAD E1α RNA did not appear to significantly change in response to changes in the level of protein intake if adequate caloric consumption was maintained either with carbohydrate or protein, but E1α RNA increased co-ordinately with

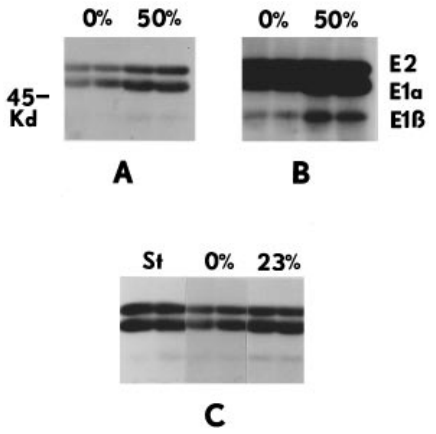

Figure 4 Changes in hepatic BCKAD subunit protein levels associated with alterations in diet

Immunoblot analysis of 100 μg protein samples of liver extracts from mice fed 0% versus 50% isocaloric protein diets (**A** and **B**) or 23% versus 0% isocaloric protein diets versus starvation for 3 days (**C**), using rabbit anti-(rat BCKAD) sera. The relative migration of E1α, E1β, and E2 subunit peptides are indicated, as is the closest molecular mass marker run in parallel. (**B**) is a longer autoradiographic exposure of (**A**) so that E1β could be visualized.

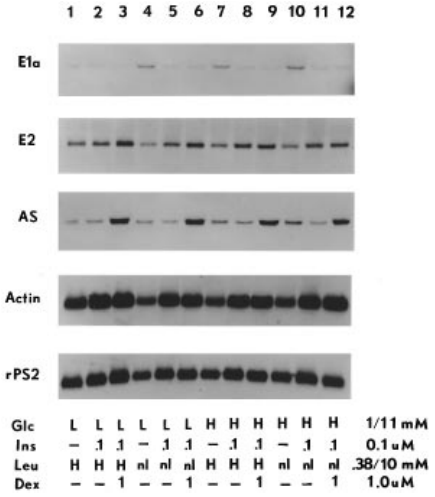

Figure 5 Alterations in BCKAD RNAs in H4IIEC3 cells

Total RNA samples (20 μg) were hybridized with the murine BCKADs indicated as well as control cDNAs for ribosomal protein S2 (rPS2), actin, and AS. Following overnight plating in RPMI + 10% fetal-calf serum (FCS), cells were grown in serum-free media for 24 h and then in glucose-free RPMI in the absence of FCS with the addition of indicated amounts of glucose, leucine, insulin and dexamethasone for 24 h: glucose ('Glc': L, 1 mM; H, 11.1 mM), leucine ('Leu': nl, 0.38 mM, H, 10.0 mM), dexamethasone ('Dex': 1 μM), insulin ('Ins': 0.1 μM). Each lane contains RNA from pooled samples from three individual plates.

E1β and E2 RNA following starvation, that is, caloric deprivation. This suggested that during starvation, different mechanisms may be involved in the control of BCKAD subunit gene expression.

To determine whether or not changes in BCKAD peptide levels were associated with changes in the BCKAD RNA, protein extracts were prepared from portions of murine livers used for RNA studies. The relative accumulation of BCKAD subunit peptides in these extracts was determined by immunoblot analysis using rabbit polyclonal antisera to E1 and E2 (an example is shown in Figure 4). Increased levels of all three BCKAD subunit peptides were detected in liver extracts in association with increased dietary protein intake (0% versus 50%). This contrasted with patterns of RNA accumulation, in which increases of E1β and E2, but not E1α, RNAs were noted (results not shown, and [20]). The magnitude of the change in the accumulation of the three peptides was similar to that observed by others in rats, suggesting that co-ordinate changes in BCKAD subunit protein occurred even when non-co-ordinated changes in BCKAD RNA were evident [19]. Following starvation, co-ordinated increases in the accumulation of both E1α and E2 peptides were observed, similar to the patterns of RNA accumulation.

A summary of the relative changes in RNA observed in livers of mice fed a variety of isocaloric diets compared with starvation is shown in Table 2. The observations of a large number of isocaloric dietary manipulations, compared with starvation, suggest that the response of BCKAD E1α RNA is non-co-ordinate with that of E1β and E2 RNAs when mice are changed from low- (6%) or 0%-protein diets to higher-protein (23%, 50%) diets which provide similar calories with alternative food groups (carbohydrates versus protein). In contrast, starvation apparently induces a different set of factors which produce a co-ordinate increase of E1α RNA with E1β and E2 RNA.

Table 2 Changes in relative levels of BCKAD RNA associated with alterations in diet

The relative differences in the densitometric values of hybridized BCKAD subunit RNAs, as well as actin and ribosomal protein S2 (rPS2), are indicated. These relative RNA levels were determined by standardization to the amounts detected in samples from mice fed 0% isocaloric protein diets, which demonstrated the lowest BCKAD RNA levels. The % protein content of the different isocaloric diets is indicated for each group of mice. The Table summarizes approximate differences observed in the accumulation of the different RNAs in three to four different experiments. Four mice per group were analysed in each experiment. *Significant changes by Student's *t*-test at the 95% confidence level are indicated.

	Relative RNA levels				
	0% Protein	6% Protein	23% Protein	50% Protein	Starved
E1α	1.0	0.7–1.0	0.8–1.0	0.8–1.0	2.0–3.0*
E1β	1.0	1.0	2.0–3.0*	2.0–3.0*	5.0–6.0*
E2	1.0	1.0	2.0–3.0*	2.0–3.0*	5.0–6.0*
Actin	1.0	1.0	1.0	1.0	1.0
rPS2	1.0	0.7–1.0	1.0	1.0	1.4–2.0

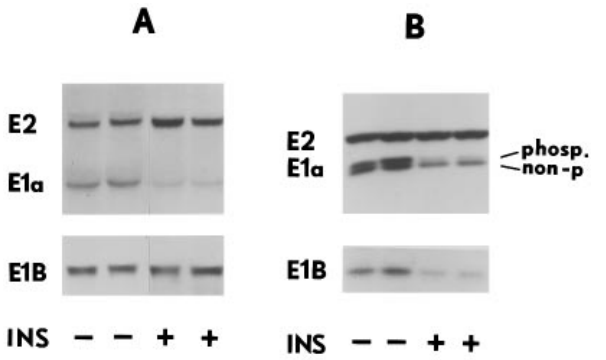

Figure 6 Effects of insulin on BCKAD subunit RNAs and protein in H4IIEC3 cells

Cells were grown for 24 h in serum-free RPMI and then in serum-free RPMI in the absence (lanes 1 and 2, ' − ') or presence (lanes 3 and 4, ' + ') of 0.1 μM insulin (INS). (**A**) Northern blot hybridization analysis of 20 μg of RNA obtained from pooled samples from three individual plates. Samples were hybridized with the BCKAD cDNAs indicated. (**B**) Protein samples (100 μg) were separated by 12% PAGE, electroblotted and analysed by immunoblot analysis with anti-BCKAD sera, as described in the Experimental section. Phosphorylated (phosp.) and non-phosphorylated (non-p) forms of E1α are indicated.

BCKAD subunit RNA expression in rat hepatoma cell line H4IIEC3

The expression of the RNA species encoding the BCKAD subunits E1α, E1β and E2 was examined in the established hepatoma cell line H4IIEC3 in order to determine if individual hormones, or normal metabolites derived from alterations in diet, played a role in their regulated expression. An example of the effects of various concentrations of glucose, leucine, insulin and glucocorticoids on the levels of BCKAD RNAs is shown in Figure 5. The only consistent finding observed was the association of depressed levels of BCKAD E1α with the presence of insulin in the medium. Of note was the finding that this effect was observed in both the presence and absence of fetal-calf serum (results not shown). Although some of the agents appeared to influence the overall levels of BCKAD RNA, the changes were either not always consistent or BCKAD specific, as determined by their effect on the control RNA species, actin and ribosomal protein S2. Insulin was associated with a lowered accumulation of BCKAD E1α RNA after both 24 and 48 h exposure (results not shown).

To further explore the effects of insulin on BCKAD E1α in these hepatoma cells, the levels of BCKAD subunit RNA and protein were simultaneously examined (Figure 6). After 24 h of exposure to insulin, E1α RNA, but not E1β and E2 RNA, decreased by 2–4-fold (based upon densitometric analysis). In the same experiment, the levels of BCKAD subunit proteins were analysed at 48 h by immunoblot analysis using polyclonal anti-E1 and anti-E2 sera. The levels of both E1α and E1β peptides decreased several-fold (3–4), but the levels of E2 remained unchanged. The two E1α bands visualized are consistent with the reported phosphorylated and non-phosphorylated forms of E1α, as described by others [11,15]. There was an apparent decrease in the relative amount of the phosphorylated versus non-phosphorylated form of E1α in association with insulin, in agreement with the observations of others in other cell systems [11]. Insulin-induced decrease in both E1α RNA and protein has been consistently observed in both the presence and absence of fetal-calf serum in H4IIEC3 cells (results not shown).

BCKAD RNA stability in H4IIEC3 cells

The apparent half-lives of the mRNAs encoding E1α and E2 in the presence and absence of insulin were determined using the transcriptional inhibitor, actinomycin D (Figure 7). The half-lives of E1α and E2 in these cells were determined to be 12.5 and 13.23 h, respectively (coefficients of correlation 0.928 and 0.998). In the presence of insulin, the levels of E1α decreased with an apparent half-life of 14.9 h (coefficient of correlation 0.935) and

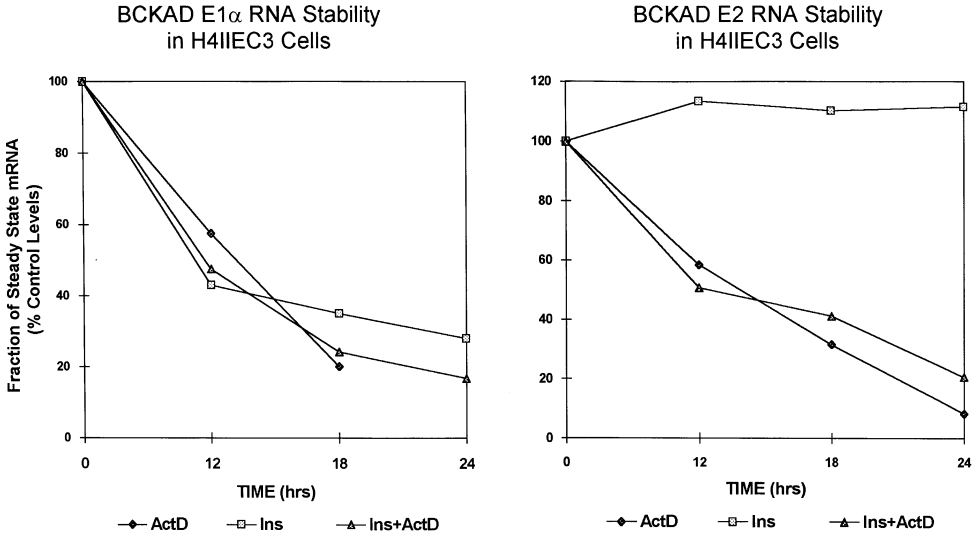

Figure 7 The effect of insulin on BCKAD E1α and E2 RNA stability in H4IIEC3 cells

RNA samples (10 μg) from pooled samples from two individual plates of cells treated with insulin (Ins; 0.1 μM) and/or actinomycin D (ActD; 2.0 μg/ml) for the indicated times were analysed by Northern blot hybridization with BCKAD E1α and E2 cDNAs. Densitometric analysis of the resultant autoradiograms is presented as the percentage of quantified signal present relative to that observed in untreated cells. All comparative densitometric analyses were performed on the same autoradiogram. Determination of the values for $t_{\frac{1}{2}}$ and coefficients of correlation given in the text were performed using linear regression analysis of the densitometric values. Note that scales on the *x*-axis are non-linear.

the levels of E2 RNA did not decrease. In the combined presence of actinomycin D and insulin, the apparent half-life of E1α RNA was slightly higher (16.4 h, coefficient of correlation 0.999), as was that for E2 (14.91 h, coefficient of correlation 0.990). These studies suggested that the changes in E1α RNA associated with insulin were not related to dramatic changes in the stability of E1α RNA. We could not directly prove that changes in E1α transcription were the basis for the insulin-induced decreases in E1α RNA since nuclear run-on studies in this cell line were not sufficiently sensitive for detecting quantitative differences in the transcription of BCKAD sequences (results not shown).

DISCUSSION

We have demonstrated that the RNA species encoding three of the core subunits of the murine BCKAD enzyme complex demonstrated differences in their patterns of accumulation in response to starvation compared with changes in dietary protein. In the liver, two of the three RNA species (E1β and E2) decreased in response to a reduction of dietary protein (23 % to 0 %) and increased in as little as 48 h in response to a dietary change from 0 % to 23 % protein (Figure 2 and [20]). In contrast, E1α RNA, as well as E1β and E2 RNAs, increased in response to starvation, suggesting that the accumulation of E1α RNA is sensitive to a set of circulating factors which are different from those present following changes in protein associated with otherwise adequate caloric diets. The isocaloric diets used in these and other experiments are expected to be insulinogenic, since both leucine (from the ingested protein) as well as glucose (from the ingested carbohydrate) are known to be stimulators of insulin secretion [33]. In contrast, during starvation, circulating insulin levels are suppressed to their lowest levels and it is during this condition that co-ordinated increases in E1α RNA with that of E1β and E2 RNAs were able to be observed. The use of the transformed hepatic cell line rat H4IIEC3 allowed the effect of insulin and other endocrine or nutritional related factors to be analysed both individually and in combination. The effect consistently observed in the majority of these conditions was the reduction of E1α RNA levels in the presence of insulin. The reduction of E1α RNA was associated with a reduction in E1α subunit protein and could not be explained by dramatic changes in the stability of the E1α RNA. Therefore, the effect of insulin on E1α RNA appears not to be at the post-transcriptional level, but direct proof of a transcriptional effect remains to be obtained. We and others have had difficulty performing direct transcriptional measurements of BCKAD sequences in a variety of established cell lines and these experiments need to await the cloning of E1α genomic sequences for more accurate, sensitive measurements.

The effect of insulin in decreasing BCKAD E1α RNA but not E1β and E2 RNAs was observed not only in the rat hepatoma cell line H4IIEC3 but also the murine hepatoma cell line Hepa 1-6 (results not shown). It was not observed in a variety of non-hepatic-type cell lines, such as fibroblasts, suggesting that it may reflect a hepatic-specific response. In pancreatic islet cells, BCKAD E1α RNA was observed to be responsive to glucose, with decreases observed with elevated levels of glucose (> 5 mM) [33]. A specific response of E1α RNA to glucose concentrations in either hepatoma cell line was not observed in our experiments, although general changes in the overall levels of many RNAs were observed with changes in glucose concentration and/or the presence of insulin. It is unclear if these changes are unique to the use of cultured cells or represent changes which in some way mimic the changes observed *in vivo*. We, and others, have previously reported that in rodents fed diets with differing amounts of protein, hepatic BCKAD E1α RNA did not change when other BCKAD subunit RNAs did change [19,20]. This may now be addressed by the possible role of insulin in suppressing BCKAD E1α RNA. Insulin release accompanies the post-prandial elevation in circulating glucose and leucine following meal ingestion. Our studies indicate that regardless of the inducer of insulin release (glucose or leucine), these diets would therefore prevent elevations in E1α RNA from being observed in hepatic cells. In contrast, during starvation, insulin levels are dramatically suppressed. Under these circumstances, E1α RNA levels are observed to increase. It is unclear whether other circulating factors also influence E1α gene expression similarly or differently from their influence on the expression of the BCKAD E1β and E2 genes. Such factors obviously play a role in the response of these latter subunits to increases in dietary protein. One possible factor, an increase in the levels of circulating glucocorticoids, is known to affect the levels of other enzymes involved in protein catabolism, such as the urea cycle enzymes AL and AS [32,35–38]. Although dexamethasone had little effect on E1α in hepatoma cells, it did under certain conditions induce higher levels of E2 RNA (Figure 5 and results not shown). Using primary hepatocytes under different cell culture conditions than ours, others have shown co-ordinated increases in all three BCKAD RNAs in response to glucocorticoids [34]. Differences in the responsiveness of the BCKAD subunit RNAs to both insulin and glucocorticoids may help explain the apparent non-co-ordinated responses of these subunits to dietary protein but the co-ordinated increase observed following starvation. With the stress of starvation, increases in circulating glucocorticoids may contribute to the increase in E2 (and E1β) and the decrease in the levels of circulating insulin may allow increases in E1α RNA to occur. It remains to be determined whether insulin directly affects the transcription of the E1α and E2 genes in similar or different fashions. These studies are currently in progress.

The mechanisms producing the variations in BCKAD RNA in response to changes in dietary protein may utilize similar factors to those responsible for the generalized increases in all three BCKAD RNAs observed during post-natal growth and development. During that time, changes in the relative ratio of insulin/glucagon as well as in circulating glucocorticoids are likely to play a role in these generalized increases in BCKAD subunit RNAs, similar to the responses observed for other genes of protein metabolism, notably AS and AL [35–37]. Both of these respond positively to glucocorticoids and in our experiments E2, but not E1α, demonstrated similar responses under some experimental conditions (Figure 5 and results not shown). Of note is that insulin may suppress the positive induction of AL RNA by glucocorticoids in fetal hepatocytes [35]. In a similar fashion, insulin may function to modulate the responses of BCKAD genes to other substances, such as glucocorticoids. This modulatory effect may be most pronounced for the E1α gene, where insulin's presence may suppress this gene's expression to minimal constitutive levels and prevent it from responding to other as yet unidentified positive regulating factors. The factor(s) during post-natal development which allow E1α RNA to increase may be similar to those which allow E1α to co-ordinately increase its RNA levels with the other BCKAD RNAs during adipocyte differentiation of 3T3-L1 fibroblasts [20,39]. All of these studies suggest that the BCKAD subunits may be similar to the urea cycle enzymes whose activities are required for sequential metabolic reactions, in that different mechanisms of regulation may be involved in the co-ordinate induction of proteins required for closely interacting enzymic activities [37,38]. The effect of insulin on BCKAD subunit E1α gene expression described in this study

is probably one of several induced by nutritional and/or other physiological stimuli to regulate homoeostasis of the BCAAs.

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