Physiol. Res. 50: 19-24, 2001

Effect of Starvation on Branched-Chain α-Keto Acid Dehydrogenase Activity in Rat Heart and Skeletal Muscle

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Received December 27, 1999
Accepted April 12, 2000

Summary

The aim of the present study was to investigate changes in the activity of branched-chain α-keto acid dehydrogenase (BCKAD) in skeletal muscle and the heart during brief and prolonged starvation. Fed control rats and rats starved for 2, 4 and 6 days were anesthetized with pentobarbital sodium before heart and hindlimb muscles were frozen *in situ* by liquid nitrogen. Basal (an estimate of *in vivo* activity) and total (an estimate of enzyme amount) BCKAD activities were determined by measuring the release of $^{14}CO_2$ from α-keto[1-^{14}C]isocaproate. The activity state of BCKAD complex was calculated as basal activity in percentages of total activity. Both basal and total activities and the activity state of the BCKAD were lower in skeletal muscles than in the heart. In both tissues, starvation for 2 or 4 days caused a decrease in the basal activity and activity state of BCKAD. On the contrary, in the heart and muscles of animals starved for 6 days a marked increase in basal activity and activity state of BCKAD was observed. The total BCKAD activity was increasing gradually during starvation both in muscles and the heart. The increase was significant in muscles on the 4th and 6th day of starvation. The demonstrated changes in BCKAD activity indicate significant alterations in branched-chain amino acid (BCAA) and protein metabolism during starvation. The decreased BCKAD activity in skeletal muscle and heart observed on the 2nd and 4th day of starvation prevents the loss of essential BCAA and is an important factor involved in protein sparing. The increased activity of BCKAD on the 6th day of starvation indicates activated oxidation of BCAA and accelerated protein breakdown.

Key words
Branched-chain amino acids • Leucine • Fasting • Protein metabolism

Introduction

Branched-chain α-keto acid dehydrogenase (BCKAD; EC 1.2.4.4) is considered the rate-limiting enzyme in the pathway of branched-chain amino acid (BCAA; valine, leucine, isoleucine) catabolism (Odessey and Goldberg 1979). BCKAD catalyzes the irreversible oxidative decarboxylation of all three branched-chain keto acids (BCKA) derived from BCAA, i.e. α-ketoisocaproate (ketoleucine), α-keto-β-methylvalerate (ketoisoleucine) and α-ketoisovalerate (ketovaline). The branched-chain acyl-CoA derivatives formed from this reaction are then dehydrogenated to form enoyl-CoA derivatives. The subsequent steps involve a series of reductions and oxidations leading to products that can enter the tricarboxylic acid cycle.

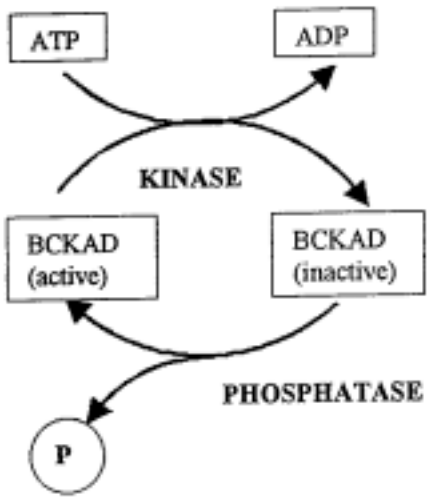

Fig. 1. *Scheme illustrating the regulation of branched-chain α-keto acid dehydrogenase (BCKAD) by phosphatase and kinase.*

BCKAD activity is controlled primarily by a phosphorylation-dephosphorylation mechanism (Fig. 1). Phosphorylation of the protein moiety, which is mediated by a specific kinase, results in inactivation of BCKAD. Dephosphorylation, mediated by a specific phosphatase, results in activation of the enzyme. The BCKAD activity is highest in the liver, intermediate in kidney and heart, and comparatively low in muscle, adipose tissue, and brain (Harper *et al.* 1984, Randle *et al.* 1984). However, when the proportion of body weight represented by each of the various tissues and organs is taken into consideration, muscles, which make up 35-40 % of total body weight, should contribute substantially to total body BCKA utilization.

In view of the important physiological functions of BCAA which include its action as substrate and regulator in protein synthesis and an essential donor of nitrogen in the synthesis of alanine and glutamine in skeletal muscles, changes in the activity of BCKAD should be of enormous importance in the protein economy of the whole body. The activity of BCKAD in skeletal muscles increases under certain circumstances, such as a high-protein diet (Block *et al.* 1987b), an exercise (Shimomura *et al.* 1990), glucocorticoid administration (Block *et al.* 1987a), insulinopenic diabetes (Paul and Adibi 1978, 1982, Aftring *et al.* 1988), endotoxemia and treatment with tumor-necrosis factor-α (TNF-α) (Nawabi *et al.* 1990, Holeček *et al.* 1996, 1997).

Considering that a characteristic response to uncomplicated starvation is a decrease in protein turnover, which enables sparing of proteins for many days, unchanged (Aftring *et al.* 1988) or even increased muscle BCKAD activities in starving animals (Goldberg and Odessey 1972, Paul and Adibi 1978), is a surprising observation. The increase in BCKAD activity enhances the catabolism of BCAA, which should under conditions of complete starvation significantly accelerate the wasting of muscle tissue by activated proteolysis, by inhibited protein synthesis, or both. It should be noted that studies evaluating the effect of starvation on BCKAD activity or BCAA oxidation in various tissues did not demonstrate a compensatory decrease in BCKAD activity or BCAA oxidation (Espinal *et al.* 1986, Solomon *et al.* 1987, Harris *et al.* 1989). In view of this controversy, the objective of the present study was to provide a new estimation of the effects of starvation on BCKAD activity in skeletal muscles and in the heart during brief as well as prolonged starvation.

Methods

Animals

Male rats (Velaz, Prague, CR) were housed in standardized cages in quarters with controlled temperature and a 12-hour light-dark cycle and received laboratory chow and drinking water *ad libitum*. All procedures were performed according to guidelines set by the Institutional Animal Use and Care Committee of Charles University.

Materials

α-keto[1-^{14}C]isocaproate was purchased from Amersham (Buckinghamshire, UK). Leucine, the sodium salt of α-ketoisocaproate, NAD^+, CoASH, dithiothreitol (DTT); thiamine pyrophosphate (TPP), N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), polyethylene glycol (PEG), disodium ethylenediamine-tetraacetic acid (Na_2EDTA), polyoxyethylene ether W-1 detergent, N-2-p-tosyl-L-lysine chloromethyl ketone (TLCK), Folin-Ciocalteu phenol reagent and albumin were purchased from Sigma Chemical (St. Louis, MO, USA). Hyamine hydroxide was obtained from Packard Instrument (Meriden, CT, USA). Remaining chemicals were from Lachema (Brno, CR).

Study design

The animals were fed *ad libitum* or starved for 2, 4, or 6 days. The enzyme assays started between 07:00 and 08:00 h to minimize the influence of diurnal variations of food intake and plasma hormone levels. Rats were anesthetized with pentobarbital sodium (5 mg/kg

body wt) and hindlimb muscles and the heart were removed as described below.

BCKAD activity

Skeletal muscles and the heart were processed for BCKAD assay by a modification of the method described by Aftring *et al.* (1986) and Block *et al.* (1987a,b). Briefly, hindlimb muscles (primarily gastrocnemius and soleus muscles) and the heart were frozen *in situ* with Wollenberger tongs precooled in liquid nitrogen and subsequently pulverized while still maintained in liquid nitrogen. BCKAD was extracted by homogenizing (1:7, W/V) the powder at 0 °C in extraction buffer containing protease, phosphatase, and kinase inhibitors (100 mM potassium phosphate buffer, 5 % polyoxyethylene ether W-1 detergent, 2 mM Na_2-EDTA, 5 mM DTT, 0.5 mM TPP, 1 mM TLCK, 20 g/ml leupeptin, 50 mM potassium fluoride (phosphatase inhibitor), and 5 mM dichloroacetate (kinase inhibitor), pH 7.4 at 0 °C) for 15 s with a Polytron homogenizer. The homogenate was centrifuged at 27 000 x g for 5 min and the supernatant fraction was saved. To this supernatant, 27 % PEG-6000 was added to give a final PEG concentration of 2 %. After stirring for 20 min on ice, the suspension was centrifuged at 12 000 x g for 10 min. To the supernatant fraction, additional 27 % PEG-6000 was added to give a final PEG concentration of 5 %. After stirring for 20 min on ice, the suspension was centrifuged as above. The PEG precipitate was suspended (1 ml/g tissue) in a buffer consisting of 25 mM HEPES, 0.2 mM Na_2-EDTA, 0.4 mM TPP, 1 mM DTT, 20 µg/ml leupeptin, and 0.2 % Brij 58 detergent, pH 7.4 at 37 °C and used as the source of BCKAD.

BCKAD activity was determined by measuring the release of $^{14}CO_2$ from α-keto[1-^{14}C]isocaproate. All assays were done in duplicate at 37 °C for 15 min in 25-ml Erlenmeyer flasks. The complete assay mixture contained, in a final volume of 1 ml, 25 mM HEPES, 0.5 mM NAD^+, 0.5 mM CoASH, 0.4 mM TPP, 0.1 mM α-keto[1-^{14}C]isocaproate (2500 DPM/nmol), 0.2 mM Na_2-EDTA, 0.2 % Brij 58 detergent, 1 mM DTT, 1 mM $MgSO_4$, 1 mM TLCK, 20 µg/ml leupeptin, and an appropriate amount of enzyme source (1-3 mg protein). At the end of 15 min of incubation, the reactions were stopped with 5 N sulfuric acid and $^{14}CO_2$ collected in methylbenzethonium hydroxide placed in center wells. After one hour of shaking, the center wells were removed and the $^{14}CO_2$ content determined by liquid scintillation spectrometry. Blanks for all assays were measured in the presence of the complete mixture supplemented with distilled water instead of enzyme source. The assay of BCKAD activity was linear up to 20 min both for initial and total BCKAD activity. Protein concentration in the enzyme source was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Basal BCKAD activity (an estimate of *in vivo* activity) was measured by using the enzyme preparation described above. Total BCKAD activity (an estimate of enzyme amount) was measured after fully activating the BCKAD by preincubating the enzyme preparation with 15 mM $MgSO_4$ for 80 min at 37 °C. The activity state of BCKA dehydrogenase complex (proportion of the enzyme present in the active state) was calculated as (basal activity/total activity) x 100.

It was not possible to express the obtained results per unit of muscle weight as the preparation of BCKAD may result in some loss of the complex during the precipitation procedure. Losses of complex would not be expected to bias measurements of the activity state of the complex because samples would be subject to the same losses. It is presumed that preincubation of the inactive form of BCKAD with Mg^{2+} fully activates the enzyme due to the action of phosphatase associated with the BCKAD complex (Paul and Adibi 1982, Aftring *et al.* 1986).

The radioactivity of the samples was measured with a liquid scintillation radioactivity counter LS 6000 (Beckman Instruments, Fullerton, CA, USA).

Statistical analysis

Results are expressed as the mean ± S.E.M. Statistical analysis was performed by Student's t-test. A difference was considered significant at $P<0.05$.

Results

Starvation caused a significant decrease in the body weight and in the weight of the gastrocnemius muscle and the heart (Table 1). The increase in relative weights of the heart and muscle is caused by a more pronounced decrease in the weight of several visceral tissues (liver, gut, etc.). As demonstrated in Tables 2 and 3, both basal and total activities of BCKAD in the skeletal muscles were lower than in the heart. Starvation for 2 or 4 days caused a decrease while starvation for 6 days led to an increase in basal activity and the activity state of BCKAD in both these tissues. Total BCKAD activity gradually increased in the course of starvation both in muscles and the heart. Significant increases were detected in skeletal muscle on the 4th and the 6th day of starvation. A significant difference in BCKAD activity was also found in the heart between the 2nd and the 6th day.

Table 1. The effect of starvation on the body weight and weights of the heart and gastrocnemius muscle.

	Control (n=5)	Duration of starvation 2 days (n = 5)	4 days (n = 5)	6 days (n = 5)
Body weight (g)				
- *beginning of starvation*	-	249 ± 2	252 ± 2	252 ± 4
- *end of starvation*	250 ± 2	209 ± 2*	186 ± 7*	163 ± 4*
Heart				
- (g)	0.69 ± 0.04	0.61 ± 0.02	0.55 ± 0.06*	0.52 ± 0.06*
- (g/kg b. w.)	2.74 ± 0.13	2.91 ± 0.08	2.94 ± 0.08*	3.09 ± 0.16
Gastrocnemius muscle				
- *(g)*	1.56 ± 0.02	1.41 ± 0.04*	1.39 ± 0.04*	1.18 ± 0.04*
- *(g/kg b. w.)*	6.23 ± 0.09	6.74 ± 0.20*	7.53 ± 0.39*	7.26 ± 0.19*

*The results are means ± S.E.M. *p<0.05 vs. control.*

Table 2. The effect of starvation on BCKAD activity in skeletal muscle.

	Control (n=8)	Duration of starvation 2 days (n = 8)	4 days (n = 7)	6 days (n = 6)
Basal activity (nmol KIC/mg protein/15 min)	0.95 ± 0.14	0.25 ± 0.05*	0.30 ± 0.04	2.57 ± 0.40*
Total activity (nmol KIC/mg protein/15 min)	20.10 ± 0.70	22.60 ± 1.64	23.49 ± 1.48*	29.31 ± 2.38*
Activity state (%)	4.81 ± 0.73	1.17 ± 0.26*	1.30 ± 0.15*	9.26 ± 1.79*

*Basal activity of BCKAD was determined before activation and total activity was achieved by preincubation with Mg^{2+} (15 mM) for 80 min at 37 °C. Activity state = (basal activity/total activity) · 100. The results are means ± S.E.M. *p<0.05 vs. control.*

Table 3. The effect of starvation on BCKAD activity in the heart.

	Control (n=8)	Duration of starvation 2 days (n = 8)	4 days (n = 7)	6 days (n = 6)
Basal activity (nmol KIC/mg protein/15 min)	2.69 ± 0.29	0.89 ± 0.11*	0.70 ± 0.11*	6.10 ± 0.47*
Total activity (nmol KIC/mg protein/15 min)	81.32 ± 3.12	80.78 ± 2.25	83.71 ± 3.18	88.72 ± 1.44
Activity state (%)	3.38 ± 0.45	1.09 ± 0.13*	0.84 ± 0.15*	6.90 ± 0.59*

*Basal activity of BCKAD was determined before activation and total activity was achieved by preincubation with Mg^{2+} (15 mM) for 80 min at 37 °C. Activity state = (basal activity/total activity) · 100. The results are means ± S.E.M. *p<0.05 vs. control.*

Discussion

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In the present study we have demonstrated changes in the catabolism of essential BCAA in skeletal muscle and in the heart during starvation. Starvation for 2 or 4 days corresponds to the phase of protein sparing, i.e. the phase in which breakdown of muscle protein is reduced, the main energy fuel are lipids and ketones, and glycerol is the main substrate in gluconeogenesis. Starvation for 6 days corresponds to the phase of protein wasting, i.e. the phase in which the sources of lipids are exhausted and amino acids become the main source of energy and the substrate for gluconeogenesis.

The decreased activities of BCKAD in muscles and the heart on the 2nd and 4th day of starvation demonstrated in this study have an important influence on protein and amino acid metabolism. At first, the decrease in BCKAD activity prevents the loss of essential BCAA, which are an essential substrate and an important regulator in the synthesis of body proteins. Then, the decreased BCKAD activity should enhance the resynthesis of BCAA from BCKA. As BCAA are an important nitrogen source for glutamine and alanine synthesis in muscle, it may be suggested that decreased activity of BCKAD helps to supply BCAA for the synthesis of alanine and glutamine. These metabolic changes induced by decreased BCKAD activity have a stimulatory effect on protein synthesis or an inhibitory effect on protein breakdown (Harper *et al.* 1984, Holeček *et al.* 2000). It may thus be suggested that the decrease in BCKAD activity is an important factor involved in protein sparing during starvation.

Unfortunately, we have found only a single study in which starvation caused a drop in the activity of BCKAD in skeletal muscle. Wagenmakers *et al.* (1984) demonstrated that starvation decreased the activity state of BCKAD in the quadriceps muscle although the actual activity of BCKAD did not change. This finding is in agreement with the observation of the gradual increase in total BCKAD activity in muscles and the heart which indicates an increasing capacity of these tissues to oxidize BCAA. We do not have a reasonable explanation for the discrepancy between the results presented in the study and the increased values of BCKAD activity in starving animals demonstrated in other laboratories (Goldberg and Odessey 1972, Paul and Adibi 1978). It should be stressed that the findings presented in this study are in agreement with a common idea about metabolic changes during starvation, especially in connection with decreased turnover of body proteins during the protein-sparing phase of starvation.

Increased activity and activity state of BCKAD on the 6th day of starvation indicate that catabolism of BCAA is accelerated. As activated oxidation of BCAA impairs the resynthesis of BCAA from BCKA, an accelerated breakdown of body proteins is necessary to cover demands of the starved body for BCAA. Progressive protein catabolism should reduce the amount of available body proteins and death may occur within a short time. It should be noted that a similar increase in muscle BCKAD activity has been demonstrated in protein catabolic states such as sepsis, trauma, cancer or after endotoxin or TNF-α treatment (Nawabi *et al.* 1990, Holeček *et al.* 1997).

A number of factors and metabolites may be important in the regulation of BCKAD kinase and phosphatase activity. BCKAD kinase is sensitive to inhibition by BCKA, especially α-ketoisocaproate (Paxton and Harris 1984) and by ADP (Lau *et al.* 1982). Other studies showed that diabetes induced by streptozotocin treatment increases BCKAD activity by inhibiting the kinase while TNF-α activates BCKAD by stimulating the phosphatase (Holeček *et al.* 1992). Block *et al.* (1987b) have demonstrated a strong correlation between ambient leucine concentrations and the activation state of skeletal muscle BCKAD in rats fed four levels of dietary protein. Furthermore, other factors may be altered in muscle cells by starvation and may modulate the activation of BCKAD (Buse *et al.* 1976, Paul and Adibi 1978, 1982). These possibilities require further investigation.

Acknowledgements

The skilful technical assistance of R. Ryšavá and H. Weisbauerová is gratefully acknowledged. This study was supported by a grant from European Society of Parenteral and Enteral Nutrition and by grant No. 306/98/0046 of the Grant Agency of the Czech Republic.

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