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Decreased enzyme activity and contents of hepatic branched-chain α-keto acid dehydrogenase complex subunits in a rat model for type 2 diabetes mellitus

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Abstract

The mitochondrial branched-chain α-keto acid dehydrogenase complex (BCKDC) is responsible for the committed step in branched-chain amino acid catabolism. In the present study, we examined BCKDC regulation in Otsuka Long-Evans Tokushima Fatty (OLETF) rats both before (8 weeks of age) and after (25 weeks of age) the onset of type 2 diabetes mellitus. Long-Evans Tokushima Otsuka (LETO) rats were used as controls. Plasma branched-chain amino acid and branched-chain α-keto acid concentrations were significantly increased in young and middle-aged OLETF rats. Although the hepatic complex was nearly 100% active in all animals, total BCKDC activity and protein abundance of E1α, E1β, and E2 subunits were markedly lower in OLETF than in LETO rats at 8 and 25 weeks of age. In addition, hepatic BCKDC activity and protein amounts were significantly decreased in LETO rats aged 25 weeks than in LETO rats aged 8 weeks. In skeletal muscle, E1β and E2 proteins were significantly reduced, whereas E1α tended to increase in OLETF rats. Taken together, these results suggest that (1) whole-body branched-chain α-keto acid oxidation capacity is extremely reduced in OLETF rats independently of diabetes development, (2) the aging process decreases BCKDC activity and protein abundance in the liver of normal rats, and (3) differential posttranscriptional regulation for the subunits of BCKDC may exist in skeletal muscle.

1. Introduction

The 3 branched-chain amino acids (BCAAs)—leucine, isoleucine, and valine—are essential components of the body proteins and are known to stimulate protein synthesis in various tissues [1]. However, BCAAs and their derivative branched-chain α-keto acids (BCKAs)—α-ketoisocaproic acid, α-keto-β-methylvaleric acid, and α-ketoisovaleric acid—are toxic when in excess and thus must be promptly degraded to avoid several morbid consequences such as those that arise during the maple syrup urine disease [2].

The first 2 steps in BCAA catabolism are common for the 3 amino acids. The first step comprises reversible transamination of BCAA, which is catalyzed by the enzyme branched-chain aminotransferase; and the second step consists of irreversible oxidative decarboxylation of BCKAs, which is catalyzed by the branched-chain α-keto acid dehydrogenase complex (BCKDC) and results in the formation of distinct acyl–coenzyme A derivatives [3]. In rats, given that skeletal muscle comprises nearly 82% of the whole-body BCAA transamination capacity and the liver is responsible for 60% to 83% of the total BCKA oxidation in the body [4,5], it has been postulated that the bulk of the excess BCKAs produced in muscles during specific nutritional and hormonal conditions is metabolized in the liver and regulated by BCKDC [6].

The BCKDC is a mitochondrial multienzyme complex that has 3 indispensable components: (1) a specific dehydrogenase (E1, $\alpha_2\beta_2$ heterotetramer, 12 per complex), (2) a specific transacylase (E2, 24-oligomer, core of the complex), and (3) dihydrolipoamide dehydrogenase (E3, homodimer, 6 per complex) [7]. Short-term regulation of the enzymatic activity of the complex occurs essentially by

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covalent modification of its E1α subunit; branched-chain α-keto acid dehydrogenase kinase (BDK) inactivates the complex by phosphorylating E1α, and a specific phosphoprotein phosphatase reactivates the complex by dephosphorylating its E1α subunit [3,8]. In addition, long-term regulation of BCKDC takes place by increases or decreases in its individual subunits [9].

Chemically induced insulinopenic diabetes has long been known to promote protein degradation in peripheral tissues, especially skeletal muscle [10-13], which results in increased circulating BCAA levels [11,12,14-16]. This, in turn, induces down-regulation in the activity and decreases in the protein content of BDK in hepatic [16,17] and muscular [18] tissues and ultimately leads to BCKDC activation [14,17,19,20]. However, some studies have found decreased BCKA decarboxylation rates in hepatocytes from diabetic rats [21] and down-regulated BCKDC in the liver during untreated streptozotocin (STZ)- [15] and alloxan- [22,23] induced diabetes. Although these discrepancies may stem from differences in the duration of uncontrolled diabetes, severity of the diabetic state, and/or nutritional condition of the animals at the time of sample collection, it appears that whole-body BCKA oxidation capacity (ie, total BCKDC activity) markedly decreases in severe ketotic diabetes induced in rats by injection of high doses of STZ [15,24] and can be nearly normalized by insulin treatment [15].

In a rat model of spontaneous type 1 diabetes mellitus [25], high levels of circulating BCAA were shown to correlate with increases in skeletal muscle BCKDC activity state, whereas total BCKDC activity tended to be lower in diabetic than in control animals [14]. Plasma concentrations of the 3 BCAAs also increase significantly in obese rats and mice [26] and in type 2 diabetes mellitus animals [27,28]. In fact, it has been reported that protein amounts of the E1α subunit are decreased in the liver and adipose tissue of obese Zucker rats [26]. In addition, very recently, we have found that hepatic BCKDC activity is significantly decreased in 2 rat models for spontaneous type 2 diabetes mellitus fed a purified diet [28]. However, BCKDC activity modulation and abundance of its component subunits in the liver and skeletal muscle of young/healthy and middle-aged/diabetic animals remain unclear. Thus, in the present study, young and middle-aged Otsuka Long-Evans Tokushima Fatty (OLETF) rats [29] were used to investigate the mechanism that regulate BCAA metabolism in vivo both before and after the onset of type 2 diabetes mellitus. As a means of saving time and limited funds, samples from the same animals used in our previous studies [30,31] were analyzed.

2. Materials and methods

2.1. Materials

Broad-specificity protein phosphatase was semipurified from rat liver by the method of Brandt et al [32]. Reagent for protein determination and goat anti-rabbit secondary antibody were purchased from Bio-Rad Laboratories (Hercules, CA). Rabbit polyclonal antisera against the E1α, E1β, and E2 subunits of BCKDC were produced in our laboratory using a standard protocol [33]. Monoclonal antibody against BDK was obtained as described previously [34]. Anti-mouse secondary antibody was purchased from Promega (Madison, WI). All other reagents were of analytical grade and were purchased from Wako (Osaka, Japan), Oriental Yeast (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma-Aldrich (Tokyo, Japan).

2.2. Animals, sample collection, and analytical methods

Animal care and sample collection and handling were conducted as described previously [30,31]. Briefly, rats were fed standard rat chow (CE-2) containing 25% protein and were dissected at 8 and 25 weeks of age after short-term (6 hours) fasting. Plasma triglycerides (TG) concentration was estimated using the Triglyceride E commercial kit purchased from Wako. Plasma BCAA concentration was assayed spectrophotometrically by recording end-point NADH (reduced form of nicotinamide adenine dinucleotide) production from the oxidative deamination of BCAAs catalyzed by leucine dehydrogenase [35,36]. Plasma BCKA concentration was assayed spectrophotometrically by recording end-point NADH production from the oxidative decarboxylation of BCKAs catalyzed by semipurified rat liver BCKDC [37]. All plasma analyses were carried out within 2 years of sample collection.

2.3. Enzyme assays

Spectrophotometric assays of the liver active BCKDC activity (ie, activity of the partially dephosphorylated enzyme or its active form in vivo), total BCKDC activity (ie, activity of the fully dephosphorylated enzyme), and BDK activity were conducted as described elsewhere [38], with a few adaptations as follow: α-chloroisocaproate final concentration in the extraction buffer was 0.1 mmol/L, dihydrolipoamide dehydrogenase final concentration in the assay buffer was 3 U/mL, and the pH of the kinase assay buffer was optimized at 7.5. In addition, centrifugations were carried out at 10 000*g* for 15 minutes; and suspending buffer was added with 100 mmol/L potassium fluoride before use. Incubation with broad-specificity protein phosphatase and $MgSO_4$ was the method chosen for BCKDC full activation [38]. *Activity unit* is defined as 1 μmol of substrate converted to product per minute at 30°C. Enzyme kinetic assays were also performed within 2 years of tissue collection; BCKDC activity has been shown to be preserved notwithstanding long-term storage (unpublished data).

2.4. Electrophoresis and immunoblotting

Protein extraction and size fractionation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, detection, and quantification of

signals on the films for the 3 subunits of BCKDC and BDK were carried out as described in our previous article [30]. After immunoblot analysis was completed, proteins immobilized on polyvinylidene difluoride membranes were stained with Coomassie Brilliant Blue G-250 to make sure that samples have been evenly loaded.

2.5. Statistical analysis

Data are presented as means ± SE. Variations between groups were analyzed by factorial analysis of variance followed by the Fisher protected least significant difference test. $P < .05$ was considered to be statistically significant. StatView for Macintosh, version 5.0, from SAS Institute (Cary, NC) was used for data analysis.

3. Results

3.1. Body weight gain and plasma biochemistry

Body weight increase in OLETF rats was faster than that in Long-Evans Tokushima Otsuka (LETO) rats, with significant difference from the start (5 weeks) to the end (25 weeks) of the study (Fig. 1). Plasma BCAA, BCKA, and TG concentrations were significantly higher in OLETF than in LETO rats of the same age, both in young and middle-aged animals (Table 1). In addition, OLETF rats aged 25 weeks had markedly higher plasma TG concentrations than OLETF rats aged 8 weeks. As we have reported [30], plasma free fatty acids concentrations were 1.6-fold higher in OLETF than in LETO rats at both 8 and 25 weeks of age. Plasma glucose concentrations were slightly, but significantly, higher in OLETF than in LETO rats only at 25 weeks of age [30]. Markedly high plasma insulin concentrations in OLETF rats aged 25 weeks confirmed that those rats were insulin resistant and developed diabetes [30].

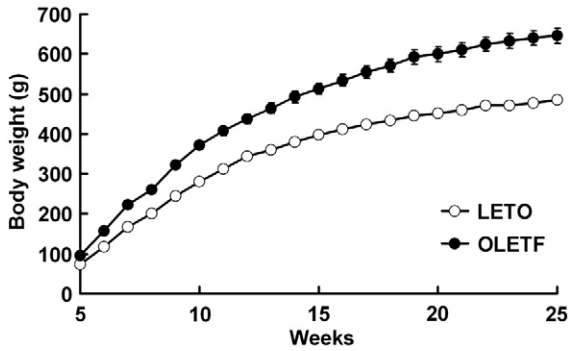

Fig. 1. Time-dependent body weight change in LETO and OLETF rats. Line graph shows the growing pattern of LETO and OLETF rats from 5 to 25 weeks of age. Data are from 12 and 6 rats in each group until the 9th week and from the 10th to the 25th week of age, respectively (means ± SE). Body weight difference between LETO and OLETF rats was statistically significant ($P < .001$) in all time points.

Table 1
Plasma BCAA, BCKA, and TG concentrations in young and middle-aged LETO and OLETF rats

	LETO		OLETF	
	8 wk	25 wk	8 wk	25 wk
Plasma BCAA (μmol/L)	259.7 ± 12.0	242.6 ± 6.5	292.2 ± 14.2*	306.6 ± 6.5‡
Plasma BCKA (μmol/L)	32.0 ± 1.7	31.3 ± 0.9	42.2 ± 2.8†	41.2 ± 1.5†
Plasma TG (mg/dL)	41.4 ± 3.1	56.4 ± 3.8	82.9 ± 7.3*	308.8 ± 20.8‡§

Plasma was obtained from blood taken just before rats were dissected 6 hours after being deprived of food. Plasma BCAA, BCKA, and TG concentrations were determined as described under "Materials and methods" (each assay was run in duplicate). Values are means ± SE (n = 6).

* $P < .05$ vs age-matched LETO.
† $P < .01$ vs age-matched LETO.
‡ $P < .001$ vs age-matched LETO.
§ $P < .001$ vs 8-week–old OLETF.

3.2. Liver BCKDC and BDK activities

Active and total BCKDC activities in the liver were very similar in each of the 4 groups of rats, showing that nearly 100% of hepatic BCKDC was in its active state at the time of dissection (Table 2). However, compared with age-matched LETO rats, OLETF rats had significantly lower enzyme activity, suggesting decreased ability to catabolyze BCKA in the liver. In addition, BCKDC activity in the liver of LETO rats aged 25 weeks was significantly lower than that in LETO rats aged 8 weeks (Table 2). Hepatic BDK activity in middle-aged OLETF rats was somewhat, but significantly, higher than that in LETO rats of the same age and in young OLETF rats (Table 2).

Table 2
Liver active BCKDC and total BCKDC activities, complex activity state, and BDK activity in young and middle-aged LETO and OLETF rats

	LETO		OLETF	
	8 wk	25 wk	8 wk	25 wk
Active BCKDC activity (U/g)	1.83 ± 0.03	1.68 ± 0.04‡	0.70 ± 0.05*	0.76 ± 0.04*
Total BCKDC activity (U/g)	1.87 ± 0.04	1.74 ± 0.04‡	0.73 ± 0.04*	0.79 ± 0.04*
BCKDC activity state (%)	98.0 ± 0.5	96.6 ± 0.9	96.2 ± 1.9	96.3 ± 1.6
BDK activity (min^{-1})	0.10 ± 0.01	0.07 ± 0.01	0.13 ± 0.02	0.30 ± 0.01*,†

The BCKDC and BDK activities in liver extracts were measured spectrophotometrically by tracking NADH production rate, as described under "Materials and methods." Enzyme activity is expressed in units per gram tissue. Values are means ± SE (n = 6).

* $P < .001$ vs age-matched LETO.
† $P < .001$ vs 8-week–old OLETF.
‡ $P < .05$ vs 8-week–old LETO.

3.3. Liver BCKDC subunits and BDK protein amounts

Comparable with BCKDC total activity levels, protein amounts of all 3 subunits of the complex (E1α, E1β, and E2) were significantly decreased in the livers of OLETF rats both at 8 and 25 weeks of age (Fig. 2A and B). Besides, middle-aged LETO rats had markedly lower hepatic BCKDC amounts than their young counterparts (Fig. 2A and B). The BDK protein abundance in hepatic tissue tended to be lower in OLETF than in LETO rats; however, owing to great variance within groups, no statistical significance was detected (Fig. 4).

3.4. Skeletal muscle BCKDC subunits and BDK protein amounts

The abundance of the BCKDC E1α subunit in skeletal muscle tended to be higher in OLETF than in LETO rats aged 8 weeks and was significantly higher in OLETF than in LETO rats aged 25 weeks (Fig. 3A and B). In contrast, E1β and E2 amounts were significantly less in OLETF than in LETO rats at both 8 and 25 weeks of age (Fig. 3A and C). The BDK protein levels in muscle were not different between OLETF and LETO rats, but were significantly

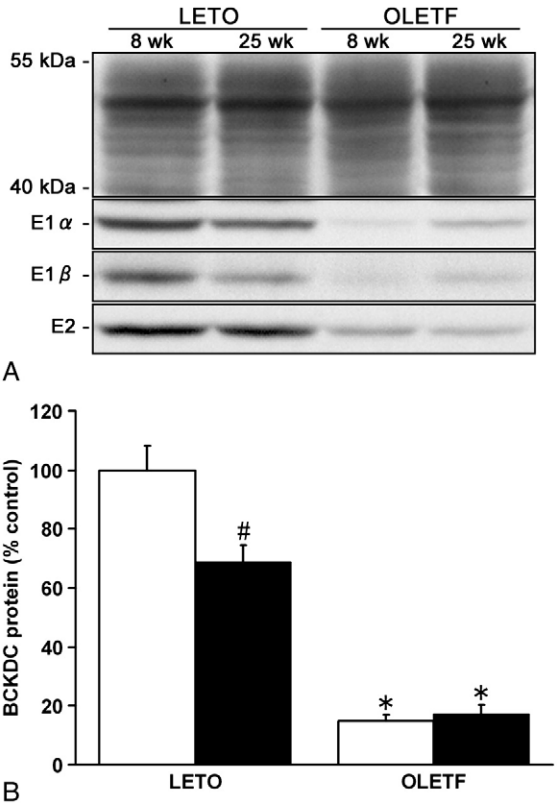

Fig. 2. Protein amounts of liver BCKDC subunits in LETO and OLETF rats. Representative immunoblots (A) and quantitative analysis (B) of hepatic BCKDC proteins in LETO and OLETF rats aged 8 (white bars) and 25 (black bars) weeks. The SDS-PAGE, Western blot analysis, and densitometry were performed as described under "Materials and methods." The upper panel in A shows representative lanes containing immobilized proteins that were stained with Coomassie Blue to demonstrate equal protein loading. Bar graph gives a quantification of the relative abundance of the 3 BCKDC subunits in the liver for 6 animals in each group (means ± SE). *$P <$ .001 vs age-matched LETO; #$P <$ .001 vs 8-week–old LETO.

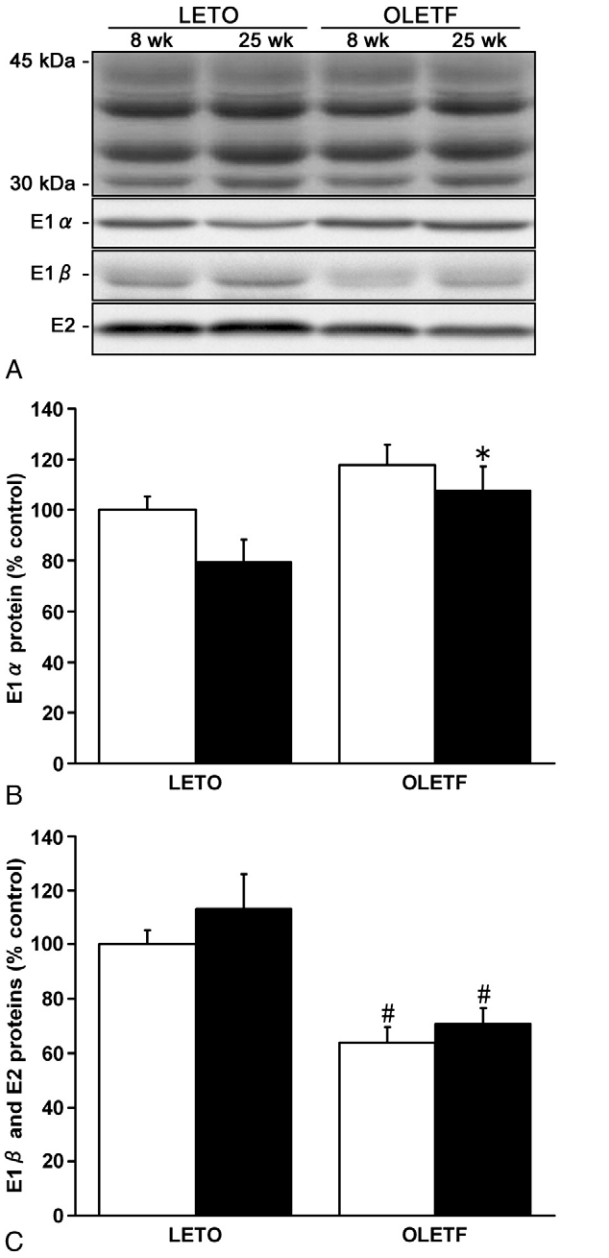

Fig. 3. Protein amounts of skeletal muscle BCKDC subunits in LETO and OLETF rats. Representative immunoblots (A) and quantitative analysis (B and C) of muscular BCKDC proteins in LETO and OLETF rats aged 8 (white bars) and 25 (black bars) weeks. The SDS-PAGE, Western blot analysis, and densitometry were performed as described under "Materials and methods." The upper panel in A shows representative lanes containing immobilized proteins that were stained with Coomassie Blue to demonstrate equal protein loading. Bar graphs give a quantification of the relative abundance of E1α (B) and E1β + E2 (C) BCKDC subunits in skeletal muscle for 6 animals in each group (means ± SE). *$P <$ .05 vs age-matched LETO; #$P <$ .01 vs age-matched LETO.

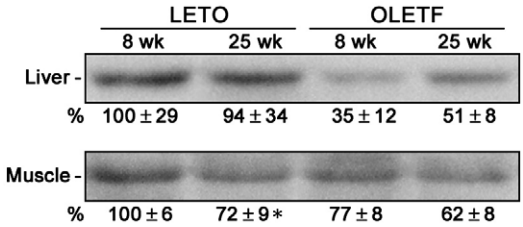

Fig. 4. Protein amounts of liver and skeletal muscle BDK in LETO and OLETF rats. Representative immunoblots of hepatic (A) and muscular (B) BDK protein in LETO and OLETF rats aged 8 and 25 weeks. The SDS-PAGE and Western blot analysis were performed as described under "Materials and methods." Values under the blots indicate the percentage abundance of BDK protein relative to LETO rats aged 8 weeks for 6 animals in each group (means ± SE). *$P < .05$ vs 8-week–old LETO.

lower in LETO rats aged 25 weeks than in LETO rats aged 8 weeks (Fig. 4).

4. Discussion

Body wasting during diabetes causes increases in the circulating levels of BCAAs and BCKAs [6], which must be committed to degradation to protect the body from their toxicity. In rats, excessive BCKA is primarily metabolized in the liver through the catalytic activity of BCKDC and other enzymes downstream the pathway [3]. In normal conditions, partially active hepatic BCKDC is expected to be up-regulated by elevations in plasma BCAA and BCKA concentrations. However, this is not always the case during experimentally induced diabetes [15,21,22], particularly in highly ketotic insulin-dependent animals. On the other hand, inactivation of the complex by phosphorylation and degradation of its components are necessary during periods of dietary protein insufficiency to conserve BCAAs for protein synthesis [6,39,40].

In the present study, no changes in the liver BCKDC activity state (percentage of BCKDC in its active, dephosphorylated state) were observed in control and experimental animals for the reason that relatively high (>25%) dietary protein from commercial chow diets keeps the complex almost fully activated in normal [24,39,41,42] and STZ diabetic rats [16,24]. However, total BCKDC activity and protein abundance of its 3 subunits were significantly decreased in the liver of OLETF rats independently of the age, indicating reduced capacity for catabolyzing the excess BCKA in these animals. Defective oxidative decarboxylation of α-keto acids has also been reported in pancreatic islets from a genetic nonoverweight type 2 diabetes mellitus rat model [43]. Furthermore, similar results have been obtained for 19-week–old OLETF and 10-week–old ZDF/CrlCrlj-Lepr^{fa} rats fed the AIN-93G rodent diet, which contains 17% protein [28]. Therefore, probably as a consequence of insulin resistance of protein metabolism that has been shown to come about in type 2 diabetes mellitus [44,45], we assume that OLETF rats are, to a certain extent, unable to cope with increasing plasma BCAA and BCKA concentrations both before and after the onset of diabetes. This hypothesis, however, calls for experimental evidence through whole-body metabolism assessment.

Total BCKDC activity and protein amounts of its subunits in the liver were significantly less in LETO rats aged 25 weeks than in LETO rats aged 8 weeks. This suggests that aging causes reduction in hepatic BCKA oxidative capacity in LETO rats. It has been reported [46] that the liver BCKDC activity increases in rat pups throughout the suckling period, reaching adult levels at the age of weaning (21 days). In addition, total BCKDC activity in the liver rises during postnatal development (until 30-40 days of age) and then tends to drop at around 60 to 70 days of age [47,48]. To our knowledge, no further information is available regarding age-dependent changes in rat hepatic BCKDC activity after 10 weeks of age; thus, this issue needs further clarification in future studies. Nevertheless, relative and absolute body fat mass noticeably increases in LETO rats from 8 to 24 weeks of age [49], indicating that, similar to the findings of She et al [26], increased body adiposity in middle-aged LETO rats may play a role in the regulation of hepatic BCKDC amount.

The whole-body relative (gram percentage) protein content in OLETF rats decreases significantly with the aging process [49], and this phenomenon may result from persistently promoted ubiquitin-proteasome–dependent proteolysis in skeletal muscle [13]. In accordance with this hypothesis, we found that skeletal muscle soluble protein concentration tended to be lower in middle-aged than in young animals (data not shown). In this study, protein abundance of 2 BCKDC subunits (E1β and E2) was decreased in muscles from OLETF rats, suggesting reduced total BCKDC activity also in this tissue. However, muscular E1α protein tended to be more plentiful in OLETF than in LETO rats, possibly attributable to the existence of distinct posttranscriptional or pretranslational regulatory mechanism for this subunit in skeletal muscle. Others have also found discrepancies in the proportion of BCKDC E1 subunits in the liver [50] and skeletal muscle [18,50] of rats during starvation [50] and STZ diabetes [18]. Notwithstanding that the importance of increased E1α protein in skeletal muscle remains unclear, this result indicates that the presence of surplus free E1α is not restricted to tissues such as the liver and kidney [23].

Hepatic BDK activity was somewhat high in middle-aged OLETF rats; however, this elevation was not sufficient to influence BCKDC activity state. In actual fact, BDK activity levels between 0.1 and 0.3 min^{-1}, equivalent to the results reported here, are extremely low and do not appear to be capable of down-regulating the complex [16,40,51]. Interestingly, plasma TG levels were very high in OLETF rats aged 25 weeks; and therefore, the possibility exists that increased BDK activity or BDK interaction with the complex [52] may coincide with high TG concentrations. Liver and muscle BDK abundance tended to be lower in OLETF than in LETO rats, with no statistical significance because of data

scattering within groups. Thus, it is challenging to understand the physiologic importance or role of BDK in the current study. The BDK gene expression in different tissues of OLETF rats both before and after the onset of diabetes is a potential topic to focus on in future research.

In summary, the present data demonstrated that OLETF rats have a very low capacity for catabolyzing α-keto acids both before and after type 2 diabetes mellitus development. In addition, aging was shown to diminish BCKDC activity and protein abundance in the liver of control rats. Decreased hepatic BCKDC total activity was accompanied by reduced amounts of its component subunits, corresponding to significantly higher circulating BCAAs and BCKAs in OLETF than in control rats. Overall, our studies suggest that suppressed carbohydrate metabolism [30,31] and altered protein metabolism in OLETF rats may be a consequence of increased body adiposity and promoted lipid metabolism.

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