Biosci. Biotechnol. Biochem., **68** (5), 1067–1072, 2004

Dietary Branched-chain Amino Acids Suppress the Expression of Pancreatic Amylase mRNA in Rats

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Received November 5, 2003; Accepted February 16, 2004

Regulators for pancreatic amylase were examined. Rats were fed *ad libitum* a 20% amino acid (AA) mixture diet (Con), a 60% AA diet (HA), a branched-chain amino acid (BCAA)-rich diet (BC), or a diet supplemented with AA other than BCAA (OA) for 7 d, or fed the Con, HA, BC diets or diets supplemented with individual BCAA. Activity and mRNA levels of pancreatic amylase in the BC and HA groups were lower than those in the Con and OA groups. Leucine and isoleucine contributed to these effects of the BC diet. The mRNA levels correlated with individual pancreatic BCAA concentrations but not with plasma insulin level. In conclusion, dietary BCAA, especially leucine and isoleucine, may reduce amylase mRNA and activity in rats.

Key words: pancreatic amylase; dietary branched-chain amino acids; dietary carbohydrate; rat

Amino acids (AA) have many physiological functions.[1–7] Recent studies have shown that some AA, especially branched-chain amino acids (BCAA), directly promote protein synthesis in pancreatic B cells[2–4] and muscles[5–7] by activation the mammalian target of rapamycin (mTOR) and also stimulate secretion of insulin by charging energy in the B cells.[8,9]

Insulin is known to up-regulate pancreatic amylase mRNA and to prevent degradation of amylase mRNA in type 1 diabetes rats.[10–13] It has also been reported that amylase activity was lowered in a model rat with insulin resistance.[14] Moreover, a mouse pancreatic amylase gene, *Amy-2.2*, has an insulin-responsive element in the 5′-flanking region.[15] These previous findings show that insulin is important for pancreatic amylase gene expression.

A high carbohydrate diet is thought to induce pancreatic amylase via insulin secretion.[16,17] However, we have previously shown that amylase activity does not always reflect carbohydrate content in the diet.[18] In our preliminary study, furthermore, ingestion of BCAA lowered pancreatic amylase activity (unpublished data) although BCAA is a stimulator of insulin secretion. Moreover, it has also been reported that exogenous insulin does not influence pancreatic amylase activity in normal rats.[19,20] These results suggest that some AA other than carbohydrate and insulin are also involved in the regulation of amylase activity in the normal rat pancreas. It is not clear, however, which AA regulates pancreatic amylase or whether insulin is involved in this regulation.

In the present study, we investigated the effect of BCAA on enzymatic activity and mRNA levels of pancreatic amylase in rats.

Materials and Methods

Animals, diets, and experimental procedure. This study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Male Wistar/ST rats (5-w-old, Japan SLC Inc., Hamamatsu, Japan) weighing approximately 100 g, were kept under a 12-h light-dark cycle and fed a semi-purified casein-based basal diet during an acclimation period. The composition of the basal diet[21] and AA mixture simulated casein[22] were described previously. Test diets in this study are shown in Table 1. All AA were kindly donated by Ajinomoto Co. (Tokyo, Japan).

Two experiments were carried out in this study. In experiment 1 (exp. 1), we investigated the contribution of BCAA in a high AA diet to inhibition of amylase activity and mRNA levels in relation to insulin action. Under a 20:00–8:00 dark-period cycle condition (as in experiment 2 (exp. 2) also), 36 acclimated rats were divided into 4 groups of 9 rats and fed a 20% AA diet (control diet, Con), a Con diet supplemented with BCAA (BC), a Con diet supplemented with AA other than BCAA (OA), or a 60% AA diet (HA) for 7 d *ad*

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Abbreviations: AA, amino acids; BCAA, branched-chain amino acids; CCK, cholecystokinin; GAPDH, glycelaldehyde-3-phosphate dehydrogenase; IRβ, insulin receptor β-subunit; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin; pY, phosphotyrosine; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1

Table 1. Composition of the Test Diets: a 20% AA Diet (Con), a Con Diet Supplemented with BCAA (BC), a Con Diet Supplemented with AA Other Than BCAA (OA), a 60% AA Diet (HA), or Con Diets Supplemented with Leucine, Isoleucine, or Valine (Leu, Ile, and Val respectively)

Diet	Con	BC	OA	HA	Leu	Ile	Val
	(g/kg diet)						
AA except BCAA[1]	161	161	483	483	161	161	161
BCAA[1]							
Leucine	17.7	53.1	17.7	53.1	53.1	17.7	17.7
Isoleucine	9.6	28.8	9.6	28.8	9.6	28.8	9.6
Valine	11.6	34.8	11.6	34.8	11.6	11.6	34.8
Corn oil	50	50	50	50	50	50	50
Mineral mixture[2]	40	40	40	40	40	40	40
Vitamin mixture[3]	10	10	10	10	10	10	10
vitamin E[4]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sucrose	695	617	373	295	660	676	672

[1] Amino acid mixture simulated casein.
[2] The mineral mixture was prepared based on the AIN-76 Workshop held in 1989. It provided (in mg/kg diet): Ca, 4,491; P, 2,997; K, 3,746; Mg, 375; Fe, 100; I, 0.32; Mn, 10.0; Zn, 34.7; Cu, 6.00; Na, 4,279; Cl, 6,542; Se, 1.05; Mo, 1.00; Cr, 0.50; B, 0.50; V, 0.25; Sn, 2.00; As, 1.00; Si, 20.0; Ni, 1.00; F, 2.72; Co, 0.20.
[3] The vitamin mixture was prepared in accordance with the AIN-76 mixture (AIN 1976) except that menadione and L-ascorbic acid were added to make a 5.82 μmol/kg diet and a 284 μmol/kg diet respectively.
[4] Vitamin E granules (Juvela, Eisai Co., Tokyo, Japan) supplied 423 μmol all-rac-α-tocopheryl acetate per kg of diet.

libitum. AA content except for BCAA in the OA diet and BCAA content in the BC diet were equal to those in the HA diet (Table 1). On day 7, the rats were killed in the period 9:00–12:00.

Experiment 2 was carried out to examine the contribution of individual BCAA to changes to amylase mRNA levels. 36 acclimated rats were divided into 6 groups of 6 rats each and given test diets that were Con, HA, BC, or Con diets supplemented with leucine (Leu), isoleucine (Ile), or valine (Val) respectively, for 7 d. The individual BCAA added to the BC, Leu, Ile, or Val diet were up to those in the HA diet (Table 1). On day 7, the rats were killed in the 9:00–12:00.

In all test diets, sucrose contents were reduced by way of supplementation of AA (Table 1).

Sample collection. On day 7, one or two pieces of the pancreas were excised under pentobarbital-anesthesia. One segment (approximate 80 mg) was homogenized in 1 ml ISOGEN (Nippon Gene Co., Tokyo, Japan) with a Polytron homogenizer (KINEMATICA, Amlehnhalde, Switzerland) to extract total RNA in exp. 1 and 2. Another segment (approximate 80 mg) was homogenized in 1 $\times$ lysis buffer for immunoblot analysis (exp. 1), as previously reported.[23] Blood was collected with an aprotinin-heparin treated syringe from the abdominal aorta (exp. 1) to assess plasma insulin levels. The rest of the pancreas was removed after killing by ensanguination from the abdominal aorta and was freeze-dried.

Sample preparation and analysis. The dried pancreas was homogenized in a buffer solution for measurement of protein content and the activity of amylase in both experiments and of chymotrypsin in exp. 1 according to the methods described previously,[21] or was homogenized in 60% acetonitrile for measurement of free AA levels in the pancreas. Free AA levels were measured by derivation of free AA into phenyl thiocarbamoyl AA by phenyl isothiocyanate and were analyzed by HPLC using the Wakopak 5C18 column with absorbance at 254 nm.[24]

Total RNA for mRNA analysis was obtained from pancreatic homogenate with ISOGEN according to the user's manual. Amylase and glycelaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified by Northern blot analysis using digoxigenin-labeled cDNA probes; the sets of sense and antisense primers for the synthesis of cDNA from the mRNA of amylase and GAPDH have been reported previously.[25]

The homogenate in the lysis buffer was immunoprecipitated with anti-insulin receptor β-subunit (IRβ) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) or anti-insulin receptor substrate 1 (IRS-1) antibody (Santa Cruz Biotechnology, Inc.) for analysis of the phosphotyrosine (pY) levels of IRβ and IRS1. The immunoprecipitant was collected with Bio-Mag goat anti-mouse IgG (Qiagen, Tokyo, Japan), resolved in a sample buffer (final concentration: 50 mmol/l Tris-HCl, 200 g/l SDS, 60 ml/l 2-mercaptoethanol, 100 ml/l glycerol, pH 7.2), subjected to immunoblot analysis, and visualized with Enhanced Chemiluminescence reagent (Amersham Bioscience, Little Chalfont, England).

Plasma was obtained from blood by centrifuge at 1,500 $\times$ g for 20 min. Plasma insulin levels were measured with radioimmunoassay (Amersham Bioscience) in duplicate according to the instructor's manual, and we confirmed the values to be accurate by repeat measurement.

Calculations. Enzymatic activity was expressed in units per mg of protein.[21] The abundances of amylase mRNA were standardized by GAPDH mRNA. The intensities of pY levels in IRβ and IRS-1 were normalized with those of IRβ and IRS-1 respectively. The value of mRNA level and those of pY levels of IRβ and IRS-1 were presented as relative values compared with those of the Con group as 1. All data were expressed as mean $\pm$ SEM. Data were analyzed by one-way ANOVA (StatView version 5.0 Software, SAS Institute Inc., Cary, North Carolina, USA) and Duncan's multiple range test. We considered $P < 0.05$ to be statistically significant.

Results

Body weight gain and food intake were not different between the groups in exp. 1 and 2. In exp. 1, pancreatic weight was higher in the BC, OA, and HA groups than the Con group. In exp. 2, pancreatic weight in the BC

Table 2. Changes in Body Weight Gain, Food Intake, and Dry Pancreas Weight in Rats

	Exp. 1[1]		
	Body weight gain	Food intake	Pancreas
	(g/day)[3]		(mg)[3]
Con	5.46 ± 0.19	13.0 ± 0.3	149 ± 4^{c}
BC	4.77 ± 0.17	12.4 ± 0.3	165 ± 6^{b}
OA	6.04 ± 0.23	13.0 ± 0.5	173 ± 4^{b}
HA	6.06 ± 0.12	12.3 ± 0.3	201 ± 3^{a}
P-value[4]	NS	NS	<0.001
	Exp. 2[2]		
	Body weight gain	Food intake	Pancreas
	(g/day)[3]		(mg)[3]
Con	4.99 ± 0.35	15.4 ± 0.6	177 ± 8^{b}
Leu	4.99 ± 0.20	16.0 ± 0.5	184 ± 8^{b}
Ile	5.16 ± 0.24	14.7 ± 0.7	195 ± 4^{b}
Val	5.01 ± 0.14	14.3 ± 0.3	202 ± 7^{ab}
BC	4.70 ± 0.40	15.0 ± 0.7	224 ± 13^{a}
HA	4.65 ± 0.08	13.7 ± 0.9	226 ± 8^{a}
P-value[4]	NS	NS	<0.001

[1] Rats were fed a 20% AA diet (Con), a Con diet supplemented with BCAA (BC), a Con diet supplemented with AA other than BCAA (OA), or a 60% AA diet (HA) for 7 d *ad libitum* in exp. 1 (n = 9).
[2] Rats were fed a Con, BC, OA, or HA diet, or Con diets supplemented with leucine (Leu), isoleucine (Ile), or valine (Val) for 7 d (n = 6).
[3] Values are mean ± SEM. Values not sharing a common superscript are significantly different ($P < 0.05$).
[4] *P*-values are analyzed by one-way ANOVA.

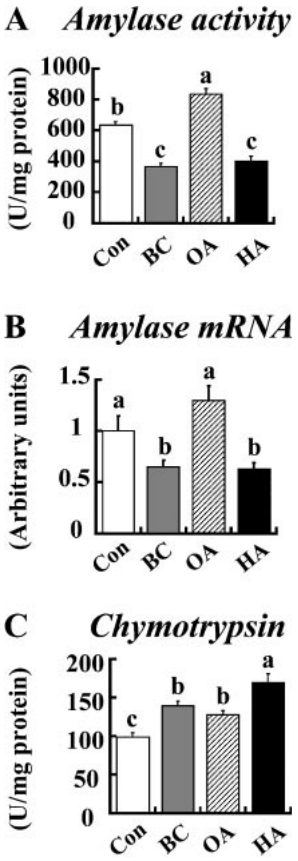

Fig. 1. Pancreatic Amylase Activity (A) and mRNA Level (B), and Chymotrypsin Activity (C) in Rats Fed a 20% AA Diet (Con), a Con Diet Supplemented with BCAA (BC), a Con Diet Supplemented with AA Other Than BCAA (OA), or a 60% AA Diet (HA) for 7 d (n = 9).

Values are mean ± SEM. The intensity of amylase mRNA was normalized by that of GAPDH mRNA, and values for the mRNA level were compared with those of the Con group as 1. Values not sharing a common letter are significantly different ($P < 0.05$).

and HA groups but not in the Leu and Ile groups was higher and that in the Val group tended to be higher than that in the Con group (Table 2).

In exp. 1, the activity and mRNA levels of amylase were lower in the BC and HA groups than in the Con and OA groups, and amylase activity in the OA group was higher than that in the Con group (Fig. 1A, B). Chymotrypsin activity was higher in the BC, OA, and HA groups than in the Con group (Fig. 1C). Phosphorylation levels of tyrosine residue in IRβ and IRS-1 were lower in the OA and HA groups and tended to be lower in the BC group compared with those in the Con group (Fig. 2B, C). Plasma insulin levels did not differ among the groups, but correlated with the pY level of IRβ ($r = 0.411$, $P < 0.05$) (Fig. 2A). Free AA concentrations in the pancreas in exp. 1 are shown in Table 3. All BCAA levels in the BC and HA groups were higher than those in the Con and OA groups. All BCAA levels correlated negatively with amylase mRNA levels (leucine, $r = -0.367$, $P < 0.05$; isoleucine, $r = -0.347$, $P < 0.05$; valine, $r = -0.463$, $P < 0.01$). Plasma AA levels were also measured in exp. 1, and the differences in BCAA levels among the groups were very similar to those in the pancreas (data not shown).

In exp. 2, amylase activity was lower in the Leu and HA groups and tended to be lower in the Ile, Val, and BC groups than that in the Con group (Fig. 3A). Amylase mRNA levels were lower in the Leu, Ile, and HA groups and tended to be lower in the Val and BC groups than in the Con group (Fig. 3B).

Discussion

In the present study, we demonstrated that dietary BCAA reduced pancreatic amylase activity associated with a reduction in amylase mRNA levels (Fig. 1A, B). In particular, dietary leucine and isoleucine may be responsible for amylase mRNA levels (Fig. 3). It has been reported that pancreatic amylase is regulated by several factors, such as insulin,[10–15] glucocorticoid,[26,27] and cholecystokinin (CCK).[25] Reduction of amylase by a low carbohydrate diet or a high protein diet has been explained by lowered insulin secretion with a reduction in dietary carbohydrate ingestion.[16,17] In this study, however, the amylase mRNA level in the BC group was as low as that in the HA group (Fig. 1A, B). The carbohydrate level in the BC diet was similar to that in the Con group, and was about 2-fold higher than that in the HA diet (Table 1). Furthermore, BCAA levels in the pancreas correlated negatively with amylase mRNA in exp. 1 (Fig. 1B and Table 3). These results indicate that pancreatic amylase activity and mRNA levels were

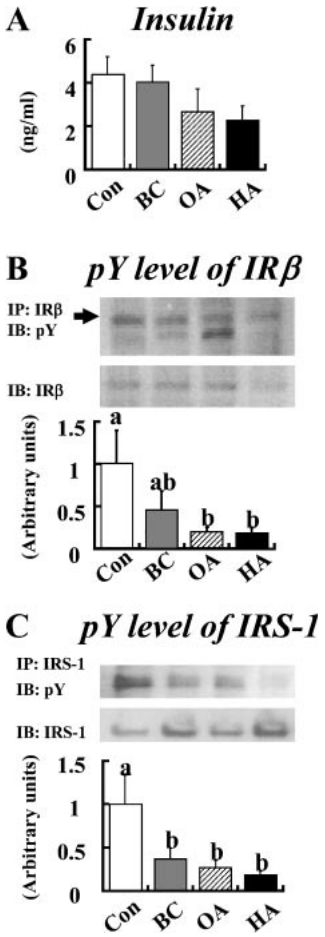

Fig. 2. Plasma Insulin Content (A) and Phosphotyrosine (pY) Level of Insulin Receptor β-Subunit (IRβ) (B) and Insulin Receptor Substrate-1 (IRS-1) (C) in Rats Fed a 20% AA Diet (Con), a Con Diet Supplemented with BCAA (BC), a Con Diet Supplemented with AA Other Than BCAA (OA), or a 60% AA Diet (HA) for 7 d (n = 9).

Values are mean ± SEM. The intensity of the pY blot was normalized with that of IRβ or the IRS-1 band in (B) and (C) respectively. Values for pY levels in IRβ and IRS-1 were compared with those for the Con group as 1. Values not sharing a common letter are significantly different ($P < 0.05$).

Table 3. Free BCAA Levels in the Pancreas in Rats Fed a 20% AA Diet (Con), a Con Diet Supplemented with BCAA (BC), a Con Diet Supplemented with AA Other Than BCAA (OA), or a 60% AA Diet (HA) for 7 d in Exp. 1[1]

	Leucine	Isoleucine	Valine
Exp. 1		(nmol/g pancreas)[2]	
Con	35.4 ± 4.7[b]	15.2 ± 2.3[b]	47 ± 4[b]
BC	85.3 ± 12.1[a]	45.1 ± 7.0[a]	119 ± 12[a]
OA	26.5 ± 4.3[b]	9.5 ± 2.3[b]	32 ± 4[b]
HA	80.1 ± 8.6[a]	36.3 ± 4.5[a]	102 ± 7[a]

[1] The respective diets were given for 7 d *ad libitum* (n = 9).
[2] Values are mean ± SEM. Values not sharing a common superscript are significantly different ($P < 0.05$).

influenced by dietary BCAA levels rather than by dietary carbohydrate levels. In many cases, a low carbohydrate diet is a high protein or a BCAA-rich

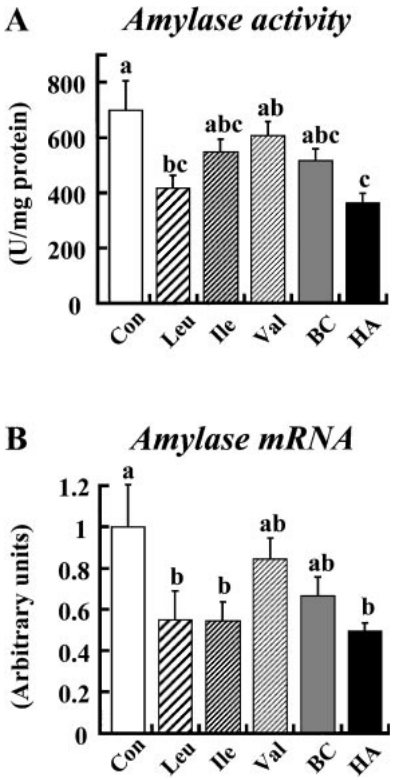

Fig. 3. Pancreatic Amylase Activity (A) and mRNA Level (B) in Rats Fed a 20% AA Diet (Con), a Con Diet Supplemented with BCAA (BC), a Con Diet Supplemented with AA Other Than BCAA (OA), a 60% AA Diet (HA), or Con Diets Supplemented with Leucine (Leu), Isoleucine (Ile), or Valine (Val) for 7 d (n = 6).

Values are mean ± SEM. The intensity of amylase mRNA was normalized with that of GAPDH mRNA, and values for the mRNA level were compared with those for the Con group as 1. Values not sharing a common letter are significantly different ($P < 0.05$).

diet.[16–23,28,29] The present findings do not conflict with previous results. BCAA may contribute to the reduction of amylase by a high protein diet. On the other hand, Amylase activity in the OA group was higher than that in the Con group and the mRNA levels showed a similar tendency (Fig. 1A, B). The mechanism is not clear. Pancreatic BCAA levels in the OA group also tended to be higher than those in the Con group, which was possibly involved in the mechanism. But BCAA levels in the BC group also tended to be higher than those in the HA group without any difference of amylase activity or mRNA level. To clarify this issue, further studies should be done.

In this study, amylase activity and mRNA levels in the OA group were not lower than those in the Con group, whereas insulin signaling was impaired in the OA group. Similarly, pY levels of IRβ and IRS-1 did not correlate with amylase mRNA levels (pY level of IRβ: $r = 0.014$, $P > 0.01$; pY level of IRS-1: $r = 0.131$, $P > 0.10$). Possibly, the pancreatic amylase mRNA level is not regulated by insulin action. In the early period of feeding, however, the plasma insulin level possibly increases in the OA group or decreases in the BC and HA groups, and this might be responsible for the

regulation of amylase mRNA levels. Although we also examined changes in insulin levels in the early stages of feeding, the relation of insulin levels in the early feeding period to pancreatic amylase activity cannot be determined due to large variation in insulin levels (data not shown). Because exogenous insulin[10–12] and endogenous insulin from transplanted B cells[13] restored amylase mRNA levels to a great extent in diabetic rats, insulin is probably important for the maintenance of amylase activity. But it has also been reported that exogenous insulin does not stimulate pancreatic amylase induction in normal rats.[19,20] These controversial results might be explained by the fact that basal insulin secretion, diminished in diabetic rat, is necessary and sufficient to express the amylase gene in normal rats. These previous findings and our results in exp. 1 suggest that pancreatic amylase gene expression depends on dietary BCAA but not on dietary carbohydrate nor, possibly, on plasma insulin levels in normal animals.

Impairment of the insulin signaling pathway has been discovered in some cases.[30–33] In some of these cases, this may be due to a lowering of insulin secretion, and others of these cases were reported as inhibition of pY levels of IRβ under hyperglycemia and long-term stimulation of tumor necrosis factor α,[30] or inhibition of pY levels of IRS-1 by phosphorylation of serine residue of IRS-1 under excess activation of mTOR.[31–33] In exp. 1, there was no difference in insulin levels between the groups, and in the BC, OA, and HA groups, pY levels of IRβ and IRS-1 were lower or tended to be lower than those in the Con group (Fig. 2). The ratios of sucrose content in the BC, OA, and HA diets to that in the Con diet were 0.89, 0.57, and 0.42 respectively, while the ratios of the insulin level in the BC, OA, and HA groups to that in the Con group were 0.92, 0.61 and 0.52 respectively. Plasma insulin levels may be dependent on sucrose content in the respective diets. As for pY levels of IRβ, they correlated with insulin levels and may be responsible for insulin level ($r = 0.411$, $P < 0.05$). The pY level of IRS-1, however, did not correlate with the insulin level or the pY level of IRβ. There is an mTOR activation pathway via IRS-1,[4,31–33] leading to protein synthesis. In some reports, long-term stimulation of mTOR leads to inhibition of IRS-1 activity and degraded IRS-1,[31–33] which perhaps means negative feedback regulation of excess stimulation of the mTOR pathway via IRS-1. BCAA, of which much was included in the BC and HA diets, may also stimulate mTOR and perhaps inhibited IRS-1 activation in the BC and HA groups. But in the OA group, which ingested less BCAA than the HA group, the pY level of IRS-1 was similar to that in the HA group. Ingestion of BCAA at the levels in the BC and HA diets may not weaken the pY of IRS-1. Even though BCAA in the BC and HA diets suppressed the pY levels of IRS-1, it may be independent of amylase gene expression.

Some hormones regulate amylase gene expression,[10–15,25–27] and our previous study showed that hyperCCKemia suppressed pancreatic amylase.[25] But other studies have shown that the adaptation of pancreatic enzymes to a high AA diet did not depend on CCK,[21,22] and in the case of this study, CCK was perhaps not involved in the suppression of amylase by BCAA.

BCAA has some physiological functions.[2–9] Leucine promotes protein synthesis via activation of mTOR in some tissues.[2–7] As shown in Fig. 1C, the BC diet induced pancreatic protease to a certain extent. Our previous study suggested that mTOR activation as estimated by 4E-BP1 phosphorylation is involved in pancreatic protease induction.[23] BCAA is not catalyzed in the liver,[34] so most of the BCAA in the blood stream might pass through the liver and be supplied to other organs, including the pancreas, in which BCAA levels in the plasma may reflect protein levels in diets more directly than the other AA and possibly acts as a signal of ingestion of high amounts of protein. Perhaps dietary BCAA regulates both suppression of pancreatic amylase mRNA levels and induction of pancreatic protease at the translation stage via the mTOR pathway.

In conclusion, dietary BCAA, especially leucine and isoleucine, down-regulate amylase mRNA levels in the rat pancreas. The relation of insulin levels to pancreatic amylase activity, however, remains unclear.

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