Branched-Chain Amino Acid-Containing Dipeptides, Identified from Whey Protein Hydrolysates, Stimulate Glucose Uptake Rate in L6 Myotubes and Isolated Skeletal Muscles

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Summary In earlier studies we showed that dietary whey protein increased skeletal muscle and liver glycogen content in exercise-trained rats. However, little is known about whether ingredients of whey protein stimulate skeletal muscle glycogen accumulation. The aim of this study was to identify bioactive peptides in whey protein hydrolysates (WPH) which stimulated glucose uptake and glycogen synthesis rate in skeletal muscles. Branched-chain amino acid (BCAA)-containing dipeptides in WPH were identified using LC/MS/MS. L6 myotubes and isolated epitrochlearis muscles were used for the glucose uptake assays. The myotubes and muscles were incubated with or without 1 mM dipeptides, LY294002 a phosphoinositide 3-kinase (PI3-kinase) inhibitor, or GF102903X an atypical protein kinase C (aPKC) inhibitor, followed by measurement of 2-deoxyglucose uptake. Isolated muscles were incubated for 3 h with or without 1 mM Ile-Leu to determine glycogen synthesis rate. The BCAA-containing dipeptides, Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu, and Leu-Leu were detected in the WPH by LC/MS/MS. These dipeptides caused significant stimulation in glucose uptake rate in the L6 myotubes. Ile-Leu, the main component in WPH, also stimulated glucose uptake in isolated skeletal muscles. Stimulation of glucose uptake by Ile-Leu was completely inhibited by treatment with either LY294002, or GF109203X in both L6 cells and isolated muscles. Ile-Leu increased glycogen contents in isolated muscles. These results suggest that BCAA-containing bioactive dipeptides in WPH stimulate glucose uptake in skeletal muscles via the PI3-kinase and aPKC pathways, resulting in increased skeletal muscle glycogen contents.

Key Words whey protein hydrolysates, bioactive peptides, glucose uptake, glycogen contents, skeletal muscle

It is well known that low- or moderate-intensity exercise has an insulin-like effect on glucose uptake in skeletal muscles (1, 2). In the absence of insulin, in vitro twitch or tetanic contractions by electrical stimulation cause an increase in muscle glucose uptake (3, 4). Furthermore, exercise training improves glucose tolerance and insulin action in subjects with either insulin-resistance or Type 2 diabetes (5–7). The molecular mechanism responsible for enhanced glucose uptake may be increased expression and/or activity of key signaling proteins involved in the regulation of glucose uptake and metabolism in skeletal muscles (8). Although it is well established that exercise-training is an effective means of maximizing glucose uptake in skeletal muscles (9), there is less information from nutritional studies on whether dietary components, such as protein have beneficial effects on this process.

Certain dietary supplements are known to modulate carbohydrate metabolism in skeletal muscles (10–13). Branched-chain amino acids (BCAAs) are essential amino acids and the major nitrogen source in these muscles. An earlier study in isolated soleus muscles of rats demonstrated that leucine stimulated glucose uptake independently of insulin (14). We have also shown dietary whey protein, which is the main source of protein in dietary supplements, increased glycogen content in liver (12) and skeletal muscles in rats (11). Whey protein contains high concentrations of BCAAs (22.3%), compared to either casein (20.3%), soy protein (17.5%) or wheat gluten (14.1%). However, it remains unclear whether ingredients of whey protein stimulate accumulation of glycogen in skeletal muscles.

Approximately 30 y ago, studies on protein digestion in the human small intestine revealed that the main products of protein digestion in the gut lumen were not single amino acids but rather di- and tri-peptides (15, 16). Recently, the intestinal oligopeptide transporter “Pept-1” was cloned in the intestines of humans and...
experimental animals (17, 18). This led to the demonstration that di- and tri-peptides are actively and rapidly taken up by enterocytes via PepT-1. Flotz et al. (19) also reported that certain tri-peptides from enriched milk beverages were absorbed into the circulation as intact forms in humans. We therefore hypothesized that as dietary protein is absorbed as amino acids, and also di-, and tri-peptides, it follows that certain BCAA-containing bioactive peptides derived from whey protein may stimulate skeletal muscle glucose uptake, resulting in an increase in skeletal muscle glycogen levels. The aim of this study was to identify BCAA-containing bioactive dipeptides in whey protein hydrolysates that stimulated glucose uptake and glycogen synthesis rate in skeletal muscles.

MATERIALS AND METHODS

Enzymatic hydrolysis of whey protein. Hydrolysis of whey protein (Tatua Co-Operative Dairy Company Limited, Morrinsville, New Zealand) (50 g/L) was carried out by incubation for 6 h at 50°C with protease enzymes (1 : 100, enzyme-to-substrate ratio, w/w) from Bacillus subtilis and Aspergillus oryzae (Amano Enzyme Inc., Nagoya, Japan). The enzymatic reactions were stopped by heating at 80°C for 10 min. The hydrolysates were spray dried and the samples dissolved in 10% acetonitrile (v/v) for use in the LC/MS/MS analyses.

Identification of branched chain amino acid (BCAA)-containing dipeptide from whey protein hydrolysates. BCAA-containing dipeptides were identified by LC/MS/MS systems (Quatro premier XE, Waters Corporation, Milford, MA, USA). All the analyses were performed on a 2.1×50 mm column with a particle size of 1.7 μm (ACQUITY UPLC BEH C18, Waters Corporation). The mobile phase A consisted of 0.05% trifluoro acetic acid (TFA) in Milli-Q water, while the mobile phase B consisted of 0.05% TFA in acetonitrile. The initial eluent composition was 100% A, followed by an increase to 40% B for 9.0 min. 80% for 1.0 min and then reduction to 100% A for 3.0 min. Total running time was 12.0 min, the eluent flow was 0.3 mL/min and the column temperature was set at 40°C. The UV trace was recorded at 215 nm.

Mass spectrometric conditions. The analytes were detected using electrospray ionization in the positive mode. Multiple-reaction-monitoring (MRM) was performed using characteristic fragmentation ions (m/z 245.1→86.1) for Ile-Ile, Ile-Leu, Leu-Leu, (231.1→86.1) for Ile-Val, Leu-Val, and (231.1→72.1) for Val-Leu. The parameters for the LC/MS/MS analysis of BCAA-containing dipeptides were as follows: capillary voltage, 3.000 V; source temperature, 120°C; desolvation temperature, 400°C; desolvation gas flow, 849 L/h; cone gas flow, 48 L/h; cone voltage and collision energy set at 25 V and 15 eV, respectively.

L6 myotubes cell culture. L6 myotubes (ICRB9081) were purchased from Human Science Research Resource Bank, Osaka, Japan. L6 skeletal muscle cells were maintained at subconfluent conditions in growth media containing α-MEM with 0.045 g/mL glucose, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. The cells were maintained in a humidified 37°C incubator at ambient oxygen and 5% CO₂. After semiconfluence was observed, the cells were differentiated in growth media containing α-MEM with 0.045 g/mL glucose, 100 U/mL penicillin, 100 μg/mL streptomycin and 2% fetal bovine serum for 5 d. Cells were then cultured on 48-well culture plates (BD BioCoat™ Collagen coat 1, Becton, Dickinson and Company, NJ, USA).

Glucose uptake assay in L6 myotubes. Glucose uptake was determined as described previously (20). Cells were washed three times with Krebs-Ringer phosphate–Hepes buffer (pH 7.4). 25 mM Hepes. 118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 5 mM NaHCO₃ in 0.1% BSA). This was followed by incubation of the cells for 180 min in KRP buffer containing 1 mM Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Leu, or Leu-Leu (Kokusan Chemical Co., Ltd., Tokyo, Japan). Cells were also treated with inhibitors with or without 10 μM LY294002, a specific inhibitor of phosphoinositide 3-kinase (PI3-kinase) (21) or 6 μM GF109203X, a specific inhibitor of atypical protein kinase C (aPKC) (22) in KRP containing 1 mM Ile-Leu. Glucose uptake was measured by adding 1 mM 2-deoxyglucose in KRP buffer, followed by incubation for 10 min at 23°C. Nonspecific glucose uptake was measured by parallel incubations in the presence of 10 μM cytochalasin B, which blocks transporter-mediated glucose uptake. Uptake was terminated by washing the cells three times with 1 mL ice-cold KRPH. The cells were then lysed with 0.1 mL of 0.1 M NaOH solution, and the solution neutralized with 0.1 mL of 0.1 M HCl. The supernatant was collected by centrifugation for the assay of 2-deoxyglucose 6-phosphate (2-DG6P). The study was approved by the Animal Committee of Food & Health R&D Laboratories, Meiji Seika Kaisha Ltd., with the animals receiving care under the guidelines laid down by this committee.

Glucose uptake assay in epitrochlearis muscles. Male Wistar rats with body weights of approximately 100 g (CLEA Japan, Inc., Tokyo, Japan) were used in this study. All the rats were housed individually in temperature-controlled rooms (22°C), with a 12-h-light/-dark cycle. The study was approved by the Animal Committee of Food & Health R&D Laboratories, Meiji Seika Kaisha Ltd., with the animals receiving care under the guidelines laid down by this committee.

After 18 h starvation, the epitrochlearis muscles were dissected under sodium pentobarbital anesthesia (40 mg/kg/BW i.p.) (23). The muscles were incubated with shaking for 60 min at 30°C in 1 mL of oxygenated Krebs-Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol, and 0.1% BSA, with or without 1 mM Ile-Leu, 10 μM LY294002, or 6 μM GF109203X. The flasks were gassed continuously with 95% O₂–5% CO₂. To remove glucose from the interstitial space, the mus-
cleared muscles were placed in 3 mL of oxygenated 95% O₂. The flasks were gassed continuously with 95% O₂−5% CO₂ during the incubations. The muscles were then blotted briefly on filter paper and frozen in liquid N₂. The samples were weighed, homogenized in 0.3 M perchloric acid, and centrifuged at 3,000 × g. After centrifuging, the neutralized supernatant was collected for the measurement of 2-DG6P. Validity check during the assay was carried out by using 2 mU/mL of purified human insulin (positive control). In addition, we confirmed that Ile-Leu was stable (100 ± 5%) in sample buffer solution with Ile-Leu also increased glucose uptake in isolated muscles (Table 2). Stimulation of glucose uptake in L6 cells and isolated muscles with Ile-Leu also increased glucose uptake in isolated muscles (Table 2).

**RESULTS**

Identification of BCAA-containing dipeptides from whey protein hydrolysates using LC/MS/MS

Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and Leu-Leu were detected in whey protein hydrolysates. Whey protein hydrolysates contained 0.15 mg Ile-Val, 0.55 mg Leu-Val, 3.62 mg Val-Leu, 0.03 mg Ile-Leu, 0.16 mg Leu-Ile, 3.69 mg Ile-Leu and 1.68 mg Leu-Leu per gram of sample. The main BCAA-containing dipeptides in whey protein hydrolysates were Ile-Leu and Val-Leu. Amino acid sequence of BCAA-containing dipeptides from whey protein was shown in Table 1.

**Effect of BCAA-containing dipeptides on 2-DG uptake in L6 myotubes**

The 2-DG assay was used to examine the effect of the following synthesized BCAA-containing dipeptides on glucose uptake: Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and Leu-Leu. As shown in Fig. 1, glucose uptake rates increased significantly when stimulated by 1 mM Ile-Leu. We therefore adopted 1 mM as the optimal concentration of BCAA-containing dipeptides in subsequent experiments.

Addition of 1 mM Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and Leu-Leu to control buffer caused a significant increase in the rate of glucose uptake in L6 myotubes (Table 2). Stimulation of glucose uptake in L6 cells by dipeptides was completely inhibited by treatment with cytochalasin B (data not shown).

**Effect of Ile-Leu with various inhibitors on 2-DG uptake in L6 cells and isolated muscles**

The increase in glucose uptake induced by 1 mM Ile-Leu was inhibited completely by pre-treatment with either LY294002 or GF109203X (Fig. 2). Pretreatment with Ile-Leu also increased glucose uptake in isolated epitrochlearis muscles, similar to that seen in the cell culture study. Addition of LY294002 or GF109203X also attenuated the increase in Ile-Leu stimulated 2-DG uptake (Fig. 2).

**Table 1. Amino acid sequence of BCAA-containing dipeptides from whey protein.**

<table>
<thead>
<tr>
<th>[M+H]*</th>
<th>Sequence</th>
<th>Origin</th>
<th>Fragment</th>
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</thead>
<tbody>
<tr>
<td>231.16</td>
<td>Val-Leu</td>
<td>β-Lactoglobulin</td>
<td>92–93</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>94–95</td>
</tr>
<tr>
<td></td>
<td>Leu-Val</td>
<td>β-Lactoglobulin</td>
<td>122–123</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-Lactalbumin</td>
<td>74–75</td>
</tr>
<tr>
<td></td>
<td>Ile-Val</td>
<td>β-Lactoglobulin</td>
<td>60–61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2–3</td>
</tr>
<tr>
<td>245.18</td>
<td>Ile-Leu</td>
<td>β-Lactoglobulin</td>
<td>56–57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-Lactalbumin</td>
<td>95–96</td>
</tr>
<tr>
<td></td>
<td>Leu-Ile</td>
<td>β-Lactoglobulin</td>
<td>1–2</td>
</tr>
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<td></td>
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<td></td>
<td>71–72</td>
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<tr>
<td></td>
<td>Ile-Leu</td>
<td>β-Lactoglobulin</td>
<td>31–32</td>
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<td></td>
<td>57–58</td>
</tr>
<tr>
<td></td>
<td>Leu-Leu</td>
<td>β-Lactoglobulin</td>
<td>103–104</td>
</tr>
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</table>

**Fig. 1. Changes in 2-deoxyglucose uptake in L6 myotubes incubated at 0, 0.25, 0.5, 1.0 or 2.0 mM Ile-Leu in control buffer. Values are means±SE (n=8), *p<0.05 vs. control (no addition, 0 mM); Dunnett’s test.**
uptake (Fig. 3).

Effect of Ile-Leu on glycogen contents in isolated muscles

Addition of 1 mM Ile-Leu to the control buffer caused a significant increase in glycogen contents in isolated epitrochlearis muscles (Fig. 4).

DISCUSSION

This is the first study to show that the BCAA-containing dipeptides, Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu, and Leu-Leu, increase glucose uptake in L6 myotubes. Ile-Leu, the main BCAA-containing dipeptide in whey protein hydrolysates, also stimulated glucose uptake and glycogen synthesis rate in isolated epitrochlearis muscles.

We first showed that BCAA-containing dipeptides caused significant increases in the rate of glucose uptake in L6 myotubes. A recent study also demonstrated that leucine stimulated glucose uptake in isolated soleus muscles, and that α-ketosacaproic acid, a metabolite of leucine, promoted glucose uptake (14). In addition, isoleucine enhanced glucose consumption and uptake under insulin-free conditions, while the effect of isoleucine was greater than that of leucine in C2C12 myotubes (28). These results demonstrated that BCAAs, such as leucine and isoleucine, increase insulin-independent glucose uptake activity in skeletal muscles. Our results showed that BCAA-containing dipeptides, a molecule consisting of two BCAAs linked by a single peptide bond, also increased skeletal muscle glucose uptake. However, it was not clear which structure of BCAAs or BCAA-containing bioactive dipeptide stimulated glucose uptake in the muscles.

In this study we used two different experimental systems to measure the glucose uptake rate, L6 myotubes and isolated epitrochlearis muscles. While the assay using L6 myotubes is very useful as a screening test, Holloszy et al. (29) indicated that glucose uptake in cultured myotubes, which resemble fetal muscle, has minimal responsiveness to insulin stimulation and therefore is not a suitable model for studying regulation of glucose uptake in skeletal muscles. In this study, we also showed that glucose uptake was increased six-fold after maximal stimulation of insulin in isolated epitrochlearis muscles, although it increased only twofold in L6 myotubes (data not shown). It is therefore important to examine glucose uptake using both L6 cells and isolated skeletal muscles, in order to represent, as closely as possible, the conditions present in an in vivo model. We confirmed that Ile-Leu, the main BCAA-containing dipeptide in whey, stimulated glucose uptake in both L6 myotubes and isolated epitrochlearis muscles. This raised the possibility that other BCAA-containing dipeptides may also stimulate glucose uptake.

Table 2. Effect of BCAA-containing dipeptides on the rate of 2-deoxyglucose uptake in L6 myotubes (nmol/10 min/well).

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>Uptake Rate (nmol/10 min/well)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>100 nM insulin</td>
<td>2.12 ± 0.14*</td>
</tr>
<tr>
<td>1 mM Ile-Leu</td>
<td>1.61 ± 0.05*</td>
</tr>
<tr>
<td>1 mM Leu-Leu</td>
<td>1.75 ± 0.05*</td>
</tr>
<tr>
<td>1 mM Ile-Ile</td>
<td>1.88 ± 0.05*</td>
</tr>
<tr>
<td>1 mM Leu-Val</td>
<td>1.73 ± 0.04*</td>
</tr>
<tr>
<td>1 mM Val-Leu</td>
<td>1.76 ± 0.07*</td>
</tr>
<tr>
<td>1 mM Leu-Va</td>
<td>1.74 ± 0.06*</td>
</tr>
<tr>
<td>1 mM Ile-Va</td>
<td>1.80 ± 0.07*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n=8).
*p<0.05 vs. control; Dunnett’s test.

Fig. 2. Effect of 1 mM Ile-Leu, 10 μM LY294002, or 6 μM GF109203X on the rate of 2-deoxyglucose uptake in L6 myotubes. Values are means ± SE (n=8). Means in a column with superscripts without a common letter differ, p<0.05; Tukey’s test.

Fig. 3. Effect of 1 mM Ile-Leu, 10 μM LY294002, or 6 μM GF109203X on the rate of 2-deoxyglucose uptake in isolated epitrochlearis muscles. Values are means ± SE (n=8). Means in a column with superscripts without a common letter differ, p<0.05; Tukey’s test.

Fig. 4. Effect of 1 mM Ile-Leu on glycogen contents in isolated epitrochlearis muscles. Values are means ± SE (n=7). *p<0.05 vs. control (no addition); Student’s t test.
in isolated skeletal muscles.

The signaling pathway for insulin-stimulated glucose uptake is well known. Tyrosine phosphorylation of the insulin receptor substrate-1 by insulin activated PI3-kinase leads to subsequent activation of downstream signaling molecules, such as protein kinase B (PKB/Akt) (30) and aPKCs (PKCα and ζ) (31, 32). In addition, numerous studies have suggested that aPKCs may also play a role as downstream targets for the IRS-PI3-kinase signaling pathway during insulin-induced GLUT4 translocation (33). In this study, an increase in glucose uptake by 1 mM Ile-Leu was inhibited completely by pre-treatment with LY294002 and GF109203X, suggesting that increased glucose uptake by Ile-Leu may be caused by aPKCs downstream of PI3-kinase.

Nishitani et al. (14) confirmed that increased glucose uptake by leucine was completely inhibited by pre-treatment with LY294002 and GF109203X. Leucine failed to stimulate PKB/Akt, indicating that signaling pathways activated by insulin and growth factors may not be necessary or present at sufficiently high levels to mediate the effects of amino acids on glucose uptake (34, 35). These authors therefore considered the downstream signal different from that of insulin-stimulated glucose uptake. These findings were similar to our results on Ile-Leu-mediated glucose uptake in both L6 myotubes and isolated muscles. Taken together, these results raise the possibility that BCAA-containing dipeptides may activate glucose uptake via the PI3-kinase and aPKC pathways, which is different from the mechanism of GLUT4 translocation by insulin.

In our previous reports, dietary whey protein increased glycogen content in both skeletal muscle and liver of exercise-trained rats to a greater extent than casein (11, 12). This finding indicates that the type of dietary protein alters glucose metabolism in both skeletal muscles and liver. This study using LC/MS/MS demonstrated for the first time that whey protein hydrolysates contained the BCAA-containing bioactive dipeptides, Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu and Leu-Leu. As shown in Table 1, β-lactoglobulin, the main component of whey protein, has numerous amino acid sequences of BCAA-containing dipeptides compared to other protein sources (36–38). Recently, Foltz et al. (19) reported that dietary tri-peptides are absorbed intact into the circulation in humans. Therefore, dietary whey protein or whey protein hydrolysates may be absorbed, not only as amino acids, but also as di- and tri-peptides, and when absorbed intact, these bioactive dipeptides may have a beneficial role in insulin independent glucose uptake in skeletal muscles. However, plasma physiological concentrations of BCAA-containing bioactive peptides have not been clear. Further study is needed to determine whether physiological levels of BCAA-containing bioactive peptides also stimulate skeletal muscle glucose uptake.

Doi et al. (28, 39) reported previously that leucine caused a significant increase of glucose incorporation into the intracellular glycogen in both in vitro and in vivo studies, whereas isoleucine did not affect glycogen synthesis, even though leucine and isoleucine were shown to stimulate insulin-independent glucose uptake in skeletal muscle cells. In a recent study using cultured human muscle cells, Armstrong et al. (40) showed that amino acids stimulated p70S6 kinase and caused transient inhibition of glycogen synthase kinase-3 (GSK-3), thereby stimulating glycogen synthesis. Furthermore, leucine stimulates glycogen synthesis as a result of the inactivation of glycogen synthase kinase-3 in L6 cells (34). This study showed that Ile-Leu also stimulated skeletal muscle glycogen synthesis, although their mechanism was still unclear. Therefore, Ile-Leu, as well as leucine, may stimulate glucose uptake, resulting in increased skeletal muscle glycogen contents.

We conclude that BCAA-containing bioactive dipeptides isolated from whey protein hydrolysates stimulate insulin independent glucose uptake in skeletal muscles possibly via the PI3-kinase and aPKC pathways, resulting in increased skeletal muscle glycogen contents.

REFERENCES


