Dietary whey protein increases skeletal muscle glycogen levels

ホエイタンパク質の筋グリコーゲン貯蔵促進効果

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Chronic feeding of whey protein, as compared to casein, increases skeletal muscle glycogen levels in exercise-trained rats.

Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats.

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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Effect of post-exercise co-ingestion of carbohydrate plus whey protein hydrolysates on blood biochemical parameters of amino acid and carbohydrate metabolism, and exercise performance in trained men.

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Chapter I

Background
1.1. Background

Muscle glycogen is an essential fuel for prolonged intense exercise. Depletion of glycogen stores is known to be associated with fatigue during both sprint and endurance exercise (1, 2) and therefore it is considered important to maintain adequate tissue stores of glycogen. While early research focused on means of increasing the muscle glycogen stores in preparation for competition and its day-to-day replenishment, recent research has focused on the most effective means of promoting its replenishment during the early hours of recovery (3, 4).

While it is well established that dietary carbohydrate is an effective source of tissue glycogen, studies have shown that combination of carbohydrate and protein is more effective than carbohydrate alone in replenishing muscle glycogen in the 4-hr period immediately after exercise (3-5). However, more studies have taken issue with the benefits of adding protein to carbohydrate supplement because the treatments were not isocaloric (3, 6, 7). Some studies showed that muscle glycogen resynthesis was similar when comparing a carbohydrate plus protein and equal caloric carbohydrate supplement (3, 6, 7). In contrast, more recent studies demonstrated that ingestion of carbohydrate plus protein produced greater glycogen resynthesis even when compared to isocaloric carbohydrate (4, 8).

It has been proposed that insulin is an important factor in both carbohydrate and protein metabolism. In humans, an acute physiological elevation in plasma insulin levels, especially
during conditions of hyperaminoacidemia, results in an additional increase in net in vivo muscle protein anabolism (9-11). Insulin also stimulates muscle glucose utilization by activating glucose transport, a process generally considered to be the main factor determining the rate of glycogen synthesis when substrate supply is adequate (12-14). The synergistic stimulating effects of combined intake of carbohydrate and protein on plasma insulin levels have already been reported. More recently, studies demonstrated an insulinotropic effect of protein when ingested in combination with carbohydrate in both clinical (15, 16) and sporting settings (17, 18). As a consequence of the ability of insulin and amino acids to promote post-exercise muscle protein anabolism and/or muscle glycogen synthesis, there is increasing interest in nutritional strategies that maximize post-exercise insulin levels and increase the availability of plasma amino acids.

Milk proteins are used mainly as the source of protein in dietary supplements. There is also evidence that stimulation of plasma insulin is greater after ingestion of whey protein compared to other protein sources such as cheese and milk (19). Whey protein comprises about 20% of the total bovine milk protein and consists mainly of β-lactoglobulin (50%), α-lactalbumin (25%), serum albumin (7%) and immunoglobulins (5%). Differences in insulinotropic effect between various dietary proteins are likely associated with the rise in plasma amino acids concentration following ingestion; casein is digested slowly and induces a lower but more prolonged hyperaminoacidemia, whereas whey protein, which is digested
quickly, induces higher responses of plasma amino acids (20). Thus, different digestive properties and amino acids composition of dietary protein may be associated with enhancing muscle glycogen synthesis.

However, there is only limited information on the effect of co-ingestion of carbohydrate with the different source (casein, whey protein) or degree of hydrolysis (non-hydrolysed protein, protein hydrolysates, amino acids) of dietary protein on glycogen accumulation in skeletal muscle.

This study was designed with

- The aim of comparing the **chronic effect** of different types of dietary protein on glycogen content in skeletal muscle of **exercise-trained rats**.

- The aim of comparing the effect of the source and degree of hydrolysis of dietary protein on **post-exercise** skeletal muscle glycogen repletion in **rats**.

- The aim of comparing the effect of **post-exercise** ingestion of carbohydrate alone with carbohydrate and two doses of whey protein hydrolysates (6.0 or 16.0 g/hr) on exercise performance and blood biochemical parameters of amino acid and carbohydrate metabolism during the **post-exercise phase in trained men**.
Chapter II

Study 1

Chronic feeding of whey protein, as compared to casein, increases skeletal muscle glycogen levels in exercise-trained rats.
2.1. Introduction

During both sprint and prolonged endurance exercise, the body must carefully balance the availability and fuel utilization in liver and skeletal muscle. In skeletal muscle, glucose transport and glycogen synthase activity are considered as the key regulatory factors for glycogen synthesis (21, 22) with defects in GLUT-4 and hexokinase, two major components of skeletal muscle carbohydrate metabolism, being linked to insulin resistance (23). Exercise training in rats has been shown to increase GLUT-4 (24-26) and hexokinase II gene expression and protein levels (27, 28). The major fate of skeletal muscle glucose uptake during the post-exercise period is storage as muscle glycogen, the magnitude of which correlates with the levels of skeletal muscle GLUT-4 and glycogen synthase activity (29). Glycogen repletion in the post-exercise period has been associated with enhanced insulin action, while reversal of glucose transport following exercise is correlated with muscle glycogen levels (30).

It is recognized that dietary carbohydrate is an effective source for tissue glycogen. Furthermore, recent studies showed that combination of carbohydrate and protein was more effective than carbohydrate alone in the replenishment of muscle glycogen during the 4 hr immediately after exercise (31, 32). However, it is not clear if different types of dietary protein affect glycogen content or the activity of glycoregulatory enzymes in tissues. Casein and whey protein are used mainly as the source of protein in dietary supplements.
Accordingly, the aim of the present study was to compare the chronic effect of casein and whey protein as the source of dietary protein on the content of glycogen, activity of glycoregulatory enzymes and mRNA expression in the skeletal muscle of exercise-trained rats.

2.2. Materials and methods

2.2.1. Animals

Male Sprague-Dawley rats (CLEA Japan Inc., Tokyo, Japan) were used in this study. All the rats were housed individually in temperature-controlled rooms (22°C), with light from 8:00 to 20:00 hrs and dark from 20:00 to 8:00 hrs. The study was approved by the Animal Committee of Meiji Seika Kaisha Ltd, Health and Bioscience Laboratories, with the animals receiving care under the guidelines laid down by this committee.

2.2.2. Diets

The design of the experimental diets followed the AIN-93 protocol (33) with the composition of the diets shown in Table 2-1. Casein and whey protein were used as the source of dietary protein. The protein content calculated as N concentration × 6.38 was measured using the Kjeldahl method. Casein (87.7 g crude protein/100 g) and whey protein (79.3 g
crude protein/100 g) were added as 200 g protein per 1 kg to the diets. The difference in the protein content between the two diets was compensated for by the addition of corn starch.

2.2.3. Experimental protocol

Twenty-four male Sprague-Dawley rats (n = 6/group), each with a body weight of about 100 g were allowed free access to food and water for 2 weeks. The rats were then divided into sedentary and exercise-trained groups, and were fed either the casein or whey protein diet. Rats in the exercise-trained groups swam simultaneously without a load 6 d/week for 120 min/d in a barrel filled with water maintained at 35°C to a depth of 50 cm so that the average surface area available to each animal was 170 cm$^2$. At the end of the 2 weeks of training, 15 hr after the end of the last training session, all the rats were fasted for 3 hr. Holness and Sugden (34) showed that fasting for 3 hr does not affect the glycogen content in tissues. The rats were killed between 9:00 and 10:00 hrs. Arteriovenous blood samples were collected from all the animals under ether anaesthesia, centrifuged at 3000 g for 15 min and the serum was then stored at −80°C. After blood collection, the abdominal cavity was opened and the gastrocnemius muscle were quickly excised, washed, weighed and frozen at −80°C until assay.
2.2.4. Serum analyses

Serum glucose concentration was measured using a glucose oxidase assay (35), serum insulin concentration by an ELISA kit obtained from Mercodia AB (Uppsala, Sweden) and serum glucagon level by an ELISA kit purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan).

2.2.5. Glycogen analysis

Tissue glycogen was isolated and purified by precipitation with ethanol from a digest formed by the addition of 5.3 M KOH, and then quantified by the phenol–sulphuric acid method (36).

2.2.6. Enzyme activities

Aliquots of skeletal muscle were homogenized in 0.1 M Tris-HCl buffer (pH 7.4) using a Potter-Elvhjem-type homogenizer fitted with a Teflon pestle. The homogenate was centrifuged for 30 min at 12000 g at 4°C and the supernatant was used immediately to determine the enzyme activities.

Hexokinase (EC 2.7.1.1), 6-phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) were assayed spectrophotometrically. Hexokinase was assayed according to Burcelin et al. (37) using an assay mixture containing 0.1 M Tris-HCl (pH 7.5), 5 mM MgCl₂,
5 mM ATP, 5 mM glucose, 0.4 mM NADP and 0.3 U glucose 6-phosphate dehydrogenase. The method of Karadsheh et al. (38) was used to analyse 6-phosphofructokinase activity with the assay mixture containing 50 mM Tris-HCl (pH 8.2), 1 mM fructose 6-phosphate, 1 mM ATP, 0.16 mM NADH, 1 mM EDTA, 2.5 mM dithiothreitol, 2 mM MgCl₂, 5 mM ammonium sulphate, 0.4 U aldolase, 2.4 U triosephosphate isomerase and 0.4 U glycerophosphate dehydrogenase. Pyruvate kinase was determined according to the method described by Harada et al. (39) using an assay mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgSO₄, 2 mM phosphoenolpyruvate, 2 mM ADP, 0.5 mM fructose 1,6-bisphosphate, 0.18 mM NADH and 8 U lactate dehydrogenase. Total glycogen synthase activity (EC 2.4.1.11) was measured by the method of Danforth (40) with enzyme activity assayed at pH 7.4 and 30°C in a reaction mixture containing 60 mM Tris-HCl (pH 7.4), 1.2 mM EDTA, 3 mM mercaptoethanol, 1.2 mM NaF, 7.5 mM UDP-glucose and 1.2% (w/v) glycogen. The assay was carried out in the presence of 12 mM glucose 6-phosphate in order to measure total glycogen synthase activity with the reaction being terminated by heating for 2 min in a boiling water-bath. The denatured protein was removed by centrifugation and UDP concentration in the supernatant solution was assayed enzymatically by allowing UDP to react with phosphoenolpyruvate in the presence of pyruvate kinase.

The total protein concentration of the tissue homogenate supernatant was measured using bicinchoninic acid with bovine serum albumin as the standard (41).
2.2.7. Total RNA isolation and cDNA

Total RNA was isolated from the skeletal muscle by the guanidine thiocyanate method of Chomczynski and Sacchi (42) using Isogen solution (Nippon Gene Co. Ltd, Tokyo, Japan). The extracted RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at a wavelength of 260 nm. Reverse transcription was used to produce cDNA from RNA using a first standard cDNA synthesis kit (Fermentas Inc., Hanover, MD, USA). The cDNA was stored at −80°C for subsequent analysis.

2.2.8. Quantitative real-time RT-PCR analysis

Real-time PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems). Primers and probes (TaqMan® Assays-on-Demand™ Gene Expression Products) were designed at Applied Biosystems (Foster City, CA, USA) from gene sequences obtained from GenBank (GLUT-4, NM-012751; hexokinase, NM-012735; glycogen synthase I, XM-341858). DNA amplification was carried out in 12.5 µL TaqMan Universal PCR Master Mix, 1.25 µL primer and probes, 2.5 µL cDNA and 8.75 µL RNase and DNase free water in a final volume of 25 µL/well. The samples were loaded in a MicroAmp 96-well reaction plate and then run using the ABI sequence detection system. After 2 min at 50°C and 10 min at 95°C, the plates were co-amplified by 50 repeated cycles, with each cycle
consisting of a 30 s denaturing step at 95°C and a 1 min annealing/extending step at 59°C.

Data were analysed by ABI software using the cycle threshold (C_T), a value that is calculated as the time, measured as cycle number, at which the reporter fluorescent emission increased beyond a threshold level, defined as the background number at which cDNA amplification was first detected. Fluorescent emission data were captured, and mRNA levels were quantified for each gene using the C_T value. The ΔC_T was calculated by subtracting the C_T for β-actin from the C_T for the gene of interest. The relative expression of the gene of interest was then calculated using the expression 2−ΔΔCT with the results being expressed as arbitrary units.

2.2.9. Statistics

Data were expressed as means ± SEM. Data were subjected to two-way ANOVA with post hoc analyses being carried out by Tukey's honestly significant difference test. Associations between the variables were examined using Pearson's correlation coefficient. Differences between groups were considered to be significant at P < 0.05.

2.3. Results

2.3.1. Initial body weight, food intake and body weight gain

Table 2-2 shows the changes in initial body weight, food intake, body weight gain and gastrocnemius muscle weights. Food intake and body weight gain were not different between
the casein and the whey protein diets. Exercise training for 2 weeks significantly decreased
the gain in body weight.

2.3.2. Serum glucose, insulin and glucagon

Serum glucose and serum insulin levels were significantly lower in the exercise training
groups compared with the sedentary groups. However, serum glucagon level was not affected
by exercise training. Figure 2-1 shows that the type of dietary protein had no effect on the
serum levels of glucose, insulin or glucagon.

2.3.3. Skeletal muscle glycogen

Rats receiving exercise training had significant increases in the glycogen content of
skeletal muscle compared with sedentary animals. The levels of skeletal muscle glycogen in
the whey protein group were also higher than levels in the casein group (Figure 2-2).

2.3.4. Skeletal muscle glycoregulatory enzyme activities

Hexokinase and total glycogen synthase activities were higher in exercise-trained rats than
in sedentary rats. In contrast, 6-phosphofructokinase and pyruvate kinase activities were not
altered by exercise training. However, groups fed whey protein had increased total glycogen
synthase activity and decreased 6-phosphofructokinase activity compared with the groups fed casein (Table 2-3).

2.3.5 Skeletal muscle mRNA levels

While exercise training resulted in a significant increase in mRNA levels of GLUT-4, hexokinase II and glycogen synthase I, the type of dietary protein had little effect on mRNA expression of these enzymes (Table 2-4).

2.4. Discussion

Depletion of glycogen stores has been associated with fatigue during both sprint and endurance exercises (1, 2, 43). Storage of glycogen in tissues is therefore of great importance, with dietary carbohydrate being the main source of tissue glycogen. However, it is not clear if different types of dietary protein affect glycogen content or the activity of glycoregulatory enzymes in tissues. The present study showed for the first time that the type of dietary protein affects muscle glycogen content, as exercise-trained rats fed a whey protein diet were shown to accumulate more glycogen than those fed a casein diet.

We also observed that swimming training increased the mRNA level of GLUT-4, hexokinase and glycogen synthase, and the activities of hexokinase and total glycogen synthase in skeletal muscle. A number of previous studies have provided evidence that there
is a relationship between skeletal muscle glycogen levels and glucose transport (21-28). Exercise training is known to increase GLUT-4 content and improve insulin-stimulated glucose uptake, with a single exercise session having been shown to increase the rate of insulin-stimulated glucose uptake and metabolism in skeletal muscle for 24–48 hr. This increase was attributed to an effect of exercise on translocation of the GLUT-4 glucose transporter (26, 44), hexokinase (27, 28) or glycogen synthase activity (45). Moreover, skeletal muscle glycogen accumulation has been shown to correlate with the relative proportion of glycogen synthase. In the present study, dietary whey protein induced the accumulation of skeletal muscle glycogen in exercise-trained rats, and increased glycogen synthase activity with a significant positive correlation ($r = 0.86; P < 0.001$) between skeletal muscle glycogen synthase and glycogen content being observed. However, we did not observe any effect of the type of dietary protein on GLUT-4 mRNA level, and hexokinase activity and mRNA expression. Skeletal muscle pyruvate kinase activity was similar with the casein and whey protein diets, while skeletal muscle 6-phosphofructokinase activity was lower in the whey groups than in the casein groups. We also observed a significant negative correlation between skeletal muscle 6-phosphofructokinase activity and glycogen content ($r = -0.37; P = 0.036$). Taken together these findings indicate that whey protein increases skeletal muscle glycogen content as a result of an increase in total glycogen synthase activity or a decrease in 6-phosphofructokinase activity (Figure 2-3).
The regulation of glycogenesis is also mediated by hormonal changes, especially insulin and glucagon. Zawadzki *et al.* (31) reported that addition of an intact protein to a carbohydrate-containing solution resulted in higher glycogen synthesis rates in subjects after exercise than did ingestion of carbohydrate alone. This was explained by the observed additional increase in plasma insulin concentrations after ingestion of the carbohydrate–protein mixture. Elevated insulin concentrations may lead to increased glucose uptake (46) and to an increase in glycogen synthase activity (14). However, in the present study, the serum levels of glucose, insulin and glucagon were similar with the two protein diets. Despite these similarities, we found dietary whey protein increased the activity of skeletal muscle glycogen synthase, and decreased the activities of skeletal muscle 6-phosphofructokinase compared with dietary casein. This finding implies that the effect of the whey protein diet on the activity of tissue glycolytic enzymes is not related to changes in the concentration of serum hormones.

It is established that certain amino acids have an important role in glucose uptake and glycogen synthesis in skeletal muscle, as previous studies in myoblast L6 cells (47) and cultured human muscle have shown that leucine and total amino acid concentration activates glycogen synthase via mammalian target of rapamycin (mTOR) activation (48). In addition, the significant changes we observed in this metabolism in rats fed whey protein may be related to other dietary ingredients of whey protein because whey protein is not purified
protein and consists of various proteins, such as β-lactoglobulin (50%), α-lactalbumin (25%), serum albumin (7%) and immunoglobulins (5%) (49). However, the manner in which these ingredients may exert this effect is not fully understood.

In conclusion, this is the first study to show that a diet based on whey protein increases glycogen content in skeletal muscle in exercise-trained rats. We consider that a daily intake of whey protein may play an important role in increasing muscle glycogen content in exercised-trained rats.
Table 2-1. Composition of the two protein diets (g/kg diet)

<table>
<thead>
<tr>
<th></th>
<th>Casein</th>
<th>Whey protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Casein</td>
<td>228</td>
<td>-</td>
</tr>
<tr>
<td>b Whey protein</td>
<td>-</td>
<td>252</td>
</tr>
<tr>
<td>c Vitamin mixture</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>d Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>e Mineral mixture</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>f Corn oil</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>g Corn starch</td>
<td>504.5</td>
<td>480.5</td>
</tr>
<tr>
<td>h Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>i Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

a Oriental Yeast Co., Ltd., Tokyo, Japan
b Nihon NZMP Co., Ltd., Tokyo, Japan
c AIN-93 diet. Nosan Corporation, Kanagawa, Japan
d Wako Pure Chemical Industries Ltd., Osaka, Japan
e Ajinomoto Co. Inc., Tokyo, Japan.
f Taiyo Kagaku Co. Ltd., Mie, Japan
g Nippon Beet Sugar Manufacturing Co. Ltd., Tokyo, Japan
h Asahi Kasei Corporation, Tokyo, Japan.
Table 2-2. Initial body weight, food intake and body weight gain

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body weight (g)</th>
<th>Food intake (g/14 days)</th>
<th>Body weight (g/14 days)</th>
<th>Gastrocnemius muscle weight (g/100 g B.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sed-Casein</td>
<td>101 ± 2</td>
<td>257 ± 5</td>
<td>114 ± 3</td>
<td>1.06 ± 0.02</td>
</tr>
<tr>
<td>Sed-Whey protein</td>
<td>101 ± 1</td>
<td>240 ± 7</td>
<td>111 ± 2</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>Ex-Casein</td>
<td>99 ± 2</td>
<td>249 ± 5</td>
<td>104 ± 2</td>
<td>1.09 ± 0.02</td>
</tr>
<tr>
<td>Ex-Whey protein</td>
<td>99 ± 2</td>
<td>240 ± 4</td>
<td>101 ± 3</td>
<td>1.04 ± 0.02</td>
</tr>
</tbody>
</table>

Two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>Diet</th>
<th>Exercise</th>
<th>Diet X Exercise</th>
<th>Diet X Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.990</td>
<td>0.081</td>
<td>0.896</td>
<td>0.777</td>
</tr>
<tr>
<td>F value</td>
<td>0.166</td>
<td>0.785</td>
<td>&lt; 0.001</td>
<td>0.689</td>
</tr>
</tbody>
</table>
| Data are expressed as means ± SEM.
<table>
<thead>
<tr>
<th>Group</th>
<th>Hexokinase (nmol/min/mg protein)</th>
<th>6-Phosphofructokinase (nmol/min/mg protein)</th>
<th>Pyruvate kinase (μmol/min/mg protein)</th>
<th>Glycogen synthase (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sed-Casein</td>
<td>18.5 ± 0.8</td>
<td>28.7 ± 2.1</td>
<td>5.67 ± 0.13</td>
<td>1.28 ± 0.36</td>
</tr>
<tr>
<td>Sed-Whey</td>
<td>18.2 ± 1.5</td>
<td>20.9 ± 1.0</td>
<td>6.02 ± 0.23</td>
<td>3.33 ± 0.40</td>
</tr>
<tr>
<td>Ex-Casein</td>
<td>24.6 ± 0.9</td>
<td>31.7 ± 3.5</td>
<td>6.07 ± 0.26</td>
<td>5.11 ± 0.54</td>
</tr>
<tr>
<td>Ex-Whey</td>
<td>25.4 ± 1.0</td>
<td>15.2 ± 2.4</td>
<td>5.63 ± 0.20</td>
<td>6.37 ± 0.52</td>
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</table>

Two-way ANOVA

<table>
<thead>
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<th>Factor</th>
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<th>Exercise</th>
<th>Diet X Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-value</td>
<td>0.861</td>
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<td>0.622</td>
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<td>p-value</td>
<td>0.815</td>
<td>0.592</td>
<td>0.090</td>
</tr>
<tr>
<td>p-value</td>
<td>0.002</td>
<td>&lt; 0.001</td>
<td>0.075</td>
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Data are expressed as means ± SEM.
Table 2-4. Skeletal muscle mRNA levels

<table>
<thead>
<tr>
<th>Group</th>
<th>GLUT-4</th>
<th>Hexokinase II</th>
<th>Glycogen synthase I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Arbitrary unit)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sed-Casein</td>
<td>100 ± 7</td>
<td>100 ± 10</td>
<td>100 ± 43</td>
</tr>
<tr>
<td>Sed-Whey</td>
<td>104 ± 8</td>
<td>81 ± 9</td>
<td>422 ± 139</td>
</tr>
<tr>
<td>Ex-Casein</td>
<td>150 ± 3</td>
<td>133 ± 6</td>
<td>1019 ± 220</td>
</tr>
<tr>
<td>Ex-Whey</td>
<td>139 ± 7</td>
<td>134 ± 9</td>
<td>1270 ± 433</td>
</tr>
</tbody>
</table>

Two-way ANOVA

<table>
<thead>
<tr>
<th></th>
<th>Diet</th>
<th>Exercise</th>
<th>Diet X Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.578</td>
<td>&lt; 0.001</td>
<td>0.246</td>
</tr>
<tr>
<td></td>
<td>0.311</td>
<td>&lt; 0.001</td>
<td>0.262</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>&lt; 0.001</td>
<td>0.889</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.
Figure 2-1. Effect of dietary protein on a) serum glucose, b) serum insulin, and c) serum glucagon concentrations in sedentary or exercise-trained rats. Data are expressed as means ± SEM. Means within the same vertical bar that do not share a common superscript letter were significantly different.
Gastrocnemius glycogen content

- Sedentary
- Exercise-trained

**Figure 2-2.** Effect of dietary protein on gastrocnemius muscle glycogen content in sedentary or exercise-trained rats. Data are expressed as means ± SEM.
Figure 2-3. Skeletal muscle carbohydrate metabolism in exercise-trained rats fed whey protein.
Chapter III

Study 2

Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats.
3.1. Introduction

Depletion of glycogen stores is known to be associated with fatigue during both sprint and endurance exercises (1, 2), and therefore it is considered important to maintain adequate tissue stores of glycogen. While it is well established that dietary carbohydrate is an effective source of tissue glycogen, studies have shown a combination of carbohydrate and protein is more effective than carbohydrate alone in replenishing muscle glycogen (3-5). However, there is only limited information on the effect of co-ingestion of carbohydrate with the different source (casein, whey protein) or degree of hydrolysis (non-hydrolysed protein, protein hydrolysates, amino acids) of dietary protein on post-exercise glycogen replenition in skeletal muscle.

It is well known that insulin stimulates glucose uptake in skeletal muscle, mainly by initiating GLUT-4 translocation from intracellular pools to the plasma membrane, resulting in stimulation of glycogen synthesis (50). Tyrosine phosphorylation of insulin receptor substrate-1 by insulin activates phosphoinositide 3-kinase (PI3-kinase) and induces activation of the downstream signaling molecules, protein kinase B (Akt/PKB) (51) and atypical protein kinase C (PKC) ζ and λ/ι (52). It is therefore important to examine phosphorylation of key enzymes, such as Akt/PKB and atypical PKC that regulate glucose uptake. Although it is well established that insulin is an effective means of maximizing glucose uptake in skeletal muscles, there is less information from nutritional studies on whether dietary components,
such as protein, have beneficial effects on this process. Recently, leucine and isoleucine were shown to activate skeletal muscle glucose uptake via the PI3-kinase and atypical PKC pathways, a mechanism that is different from that involved in GLUT-4 translocation induced by insulin (53-55). However, the phosphorylation state of the enzymes that comprise these pathways has not been studied in vivo following ingestion of carbohydrate and protein after exercise.

The aims of the present study were therefore to compare the effect of the source and degree of hydrolysis of dietary protein on glycogen levels in the post-exercise phase and to investigate the effects of post-exercise carbohydrate and protein supplementation on phosphorylated signaling molecules of Akt/PKB and atypical PKC, key enzymes regulating glucose uptake.

3.2. Materials and Methods

3.2.1. Animals

Male Sprague-Dawley rats with body weights of approximately 150 g (CLEA Japan, Inc., Tokyo, Japan) were used in this study. All rats were housed individually in temperature-controlled rooms (22°C), with light from 8:00 to 20:00 and dark from 20:00 to 8:00. The study was approved by the animal committee of Meiji Seika Kaisha Ltd., Food and Health R&D Laboratories, with the animals receiving care under the guidelines laid down by
3.2.2. Exercise protocol

Exercise training and glycogen depletion exercise protocols were followed by the modified procedure of Sonou et al. (56). On the first day, to become accustomed to swimming training, the rats swam without a load for 4 hr in two 2 hr sessions, separated by 30 min of rest in a barrel filled with water maintained at 35°C to a depth of 50 cm, so that the average surface area available to each animal was 170 cm$^2$. On the second and third days, the rats swam without a load for 6 hr in two 3 hr bouts, separated by 45 min of rest. Ren et al. (26) reported that this exercise training caused a two-fold increase in skeletal muscle GLUT-4 contents and mRNA levels, and insulin- and contractions-stimulated glucose uptake. On the final day, the rats swam for 4 hr with a load equivalent to 2% of body weight in order to deplete skeletal muscle glycogen levels. Our preliminary study showed that skeletal muscle glycogen level prior to glycogen depletion exercise was $12.2 \pm 2.4$ mg/g (data not shown).

3.2.3. Experimental protocol

The experimental protocol is shown in Figure 3-1. The rats had free access to food (protein 23.6%, fat 5.3%, carbohydrate 54.4%, ash 6.1%, fiber 2.9%, moisture 7.7%, MF, Oriental Yeast Co., Ltd., Osaka, Japan) and drinks. One day before the glycogen depletion
exercise, the rats were fed 5 g of a restricted diet (MF, Oriental Yeast Co., Ltd., Osaka, Japan). Immediately after the glycogen depletion exercise, one group (n = 8/group) was sacrificed and the other groups (n = 8/group) were given test solutions orally using a sonde (1.0 mL/100 g body weight (BW)). Two hrs after ingestion of the test solutions, the remaining rats were sacrificed under sodium pentobarbital anesthesia (40 mg kg/BW i.p.). Immediately after sacrifice, the triceps muscles were excised quickly, washed and then frozen at -80°C until assay.

3.2.4. Preparation of test solution

Whey protein (Tatua Co-Operative Dairy Company Limited, New Zealand), whey protein hydrolysates (Meiji Seika Kaisha Ltd., Japan), BCAAs (Ajinomoto Co., Inc., Japan) and casein hydrolysates (DSM Food Specialties, Netherland) were used. The protein content of these preparations was measured by the Kjeldahl method (57). The amino acid composition of the preparations is shown in Table 4-1. The average chain length of the peptides was calculated as the ratio of total nitrogen (41) to amino nitrogen (AN) in the protein samples. Average peptide length, calculated as the TN/AN ratio, was similar in the whey protein (TN/AN = 3.64) and casein hydrolysates (TN/AN = 4.63).

Solutions of glucose (30% (w/v)), glucose plus whey protein, whey protein hydrolysates, casein hydrolysates (30% glucose plus 10% protein) and glucose plus BCAA (30% glucose
plus 1.16% leucine, 0.54% isoleucine, 0.54% valine) were prepared. The total amount of BCAAs was similar to their content in whey protein (Table 3-1).

3.2.5. Muscle glycogen contents

Perchloric acid extracts of muscle were assayed for glycogen by the amyloglucosidase method (58).

3.2.6. Western blotting

Muscle samples (50 mg) were weighed and homogenized in ice-cold buffer pH 7.4 containing 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1% Triton-X100, 1.0 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 2.0 mM Na₃VO₄, aprotinin 10 µg/mL, leupeptin 10 µg/mL, pepstatin 0.5 µg/mL and PMSF 0.5 µg/mL. The homogenates were centrifuged at 4000 g for 30 min at 4°C and the supernatants then aliquoted into several test tubes and stored at -80°C for later analysis. The total protein concentration of the tissue homogenate supernatants was measured using bicinchoninic acid with bovine serum albumin as the standard. Protein phosphorylation was determined using western blotting. Equal amounts of muscle proteins (50 µg for phosphorylated Akt/PKB and phosphorylated PKCζ) were separated by gel electrophoresis, using sodium dodecyl sulphate (SDS)-PAGE, 5.0–10% gradient gels (Perfect NT Gel, DRC, Co., Ltd., Tokyo, Japan). The proteins were then
transferred to polyvinylidene fluoride (PVDF) membranes (Hybond-P, Amersham Biosciences, Piscataway, NJ, USA) and blotted for 1 hr at room temperature with gentle agitation in freshly prepared TBS containing 0.5% nonfat dry milk and 0.1% tris-buffered saline with tween-20. The PVDF membranes were then incubated with gentle agitation at 4°C over night with a primary antibody, which for phosphorylated Akt/PKB was rabbit anti-phospho-Akt (Ser\textsuperscript{473}) (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA), and for phosphorylated PKCζ was rabbit anti-phospho-PKCζ (Thr\textsuperscript{410}) (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the membranes were washed in 0.1% Tris-buffered saline-tween-20 solution, they were incubated with a secondary reagent for 1 hr at room temperature with agitation. An anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Beverly, MA, USA) was used at a dilution of 1:3000 for both phosphorylated Akt/PKB and phosphorylated PKCζ. The PVDF membranes were then washed in 0.1% tris-buffered saline-tween-20, and the antibody-bound proteins visualized using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Amersham, UK) according to the manufacturer’s protocol. The images were visualized using the Chemi-doc Gel Quantification System (Bio-Rad, Hercules, CA, USA)

3.2.7. Statistics

Data were expressed as means ± SEM. The significance of differences between the means
of the test and control groups was determined by one-way ANOVA and Tukey’s post hoc analyses. Associations between the variables were examined using Pearson’s correlation coefficients. Differences between groups were considered to be statistically significant at $P < 0.05$.

### 3.3. Results

#### 3.3.1. Skeletal muscle glycogen contents

Skeletal muscle glycogen content post-exercise and after 2 hr of recovery are shown in Figure 3-2. Addition of the proteins to the glucose solution, with the exception of casein hydrolysates, resulted in significant increases in muscle glycogen content, compared to glucose only. In particular, whey protein hydrolysates caused greater increases in post-exercise skeletal muscle glycogen content than whey protein, BCAA, and casein hydrolysates.

#### 3.3.2. Skeletal muscle phosphorylated Akt/PKB and PKCζ levels

To determine the effect of co-ingestion of glucose and dietary protein on phosphorylated Akt/PKB and PKCζ, the key enzymes that regulate glucose uptake, we selected the groups of glucose and glucose plus whey hydrolysates that caused maximum post-exercise stimulation of skeletal muscle glycogen repletion. Post-exercise ingestion of glucose significantly
increased phosphorylated Akt/PKB levels, compare to the immediate post-exercise levels, whereas phosphorylated PKCζ levels did not change. Ingestion of glucose plus whey protein hydrolysates after exercise significantly increased both phosphorylated Akt/PKB and PKCζ levels, compared to the immediate post-exercise and ingestion of glucose only.

3.3.3. Correlation between glycogen content and phosphorylated Akt/PKB and PKCζ in skeletal muscle

We showed there was a significant positive correlation between skeletal muscle glycogen content and levels of phosphorylated Akt/PKB and phosphorylated PKCζ.

3.4. Discussion

Several studies have reported that co-ingestion of protein with lower quantities of carbohydrate (≤ 0.8 g/kg/hr) accelerate postexercise muscle glycogen synthesis when compared to the ingestion of carbohydrate only in human (3-5, 59). However, it is not clear whether the source and degree of hydrolysis of dietary protein ingested with carbohydrate influences post-exercise glycogen accumulation in skeletal muscle. This study showed that ingestion of carbohydrate plus whey protein hydrolysates was more effective for increasing post-exercise skeletal muscle glycogen content in rats than ingestion of other protein sources.

We found that the source and hydrolysis of dietary protein affected post-exercise glycogen
repletion in skeletal muscles. Whey protein hydrolysates caused significantly greater increases in glycogen contents than either whey protein, casein hydrolysates or BCAA. Possible explanations for the increase in skeletal muscle glycogen levels are the amino acid composition and degree of hydrolysis of dietary proteins. A recent study demonstrated that amino acids, especially BCAA, stimulated glycogen synthesis in skeletal muscle. In that study, Armstrong et al. (48) used cultured human muscle cells to show that amino acids stimulated p70S6 kinase and caused transient inhibition of glycogen synthase kinase-3 (GSK-3), thereby increasing glycogen synthesis. Peyrollier et al. (47) also showed leucine stimulated glycogen synthesis in L6 cells as a result of inactivating glycogen synthase kinase-3, while Doi et al. (60) reported that leucine caused a significant increase in glucose incorporation into intracellular glycogen in vivo studies. In addition, the present study showed that skeletal muscle glycogen content following co-ingestion of glucose and BCAA reached the same level as that attained with glucose and whey protein. Taken together, these results indicate that the BCAA content, especially leucine, in whey protein plays an important role in post-exercise glycogen recovery.

However, comparison of the different types of protein showed that despite the BCAA levels in whey protein and casein hydrolysates being similar, skeletal muscle glycogen contents was different following supplementation of the two hydrolysates. Furthermore, ingestion of whey protein hydrolysates caused significantly greater increases in skeletal
muscle glycogen levels than non-hydrolysed whey protein even though their amino acid composition was the same. These results therefore indicated that another factor, other than the amino acid composition of dietary protein, contributed to post-exercise glycogen repletion. Recently, we demonstrated in an *in vitro* study that BCAA-containing bioactive peptides such as Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Ile-Leu, Leu-Ile, and Leu-Leu increased the rate of glucose uptake, resulting in accumulation of glycogen (55). Whey protein and casein hydrolysates contain 21.63 and 3.31 mg/g BCAA-containing bioactive peptides, respectively. Therefore, certain bioactive peptides, such as BCAA-containing peptides, may also contribute to enhanced post-exercise skeletal muscle glycogen levels.

Although it is well known that ingestion of protein with carbohydrate has a beneficial role on the rate of muscle glycogen storage after exercise, compared to carbohydrate alone, there is only limited *in vivo* data demonstrating how downstream signaling molecules are changed after ingestion of carbohydrate and protein drinks. This study showed that post-exercise ingestion of glucose plus whey protein hydrolysates in rats increased both Akt/PKB and PKCζ phosphorylation, compared to glucose alone.

It is possible that skeletal muscle Akt/PKB and PKCζ may be activated by both insulin-dependent and insulin-independent mechanisms. Insulin is a strong activator of muscle glycogen synthesis due to its stimulating effect on glucose transport via activation of Akt/PKB, atypical PKC and glycogen synthase. Although pancreatic insulin secretion is
regulated primarily by the concentration of blood glucose, protein as well as some amino acids also stimulates insulin secretion. In situations when protein and carbohydrate are consumed together, the insulin response is greater than that predicted by the sum of the individual responses. Furthermore, milk proteins have insulinotropic properties, with the whey fraction being a more efficient insulin secretagogue than casein (61-63). As a consequence, co-ingestion of glucose and whey protein hydrolysates stimulate insulin release to a greater extent than glucose alone, suggesting that this elevation in insulin level, may in turn, activate Akt/PKB and PKCζ in skeletal muscle.

A recent study demonstrated that certain amino acids directly activate the key proteins in the insulin signaling pathway. In that study, Nishitani et al. (53) confirmed that increased glucose uptake induced by leucine was inhibited completely by pre-treatment with LY294002, a PI-3 kinase inhibitor, and GF109203X, an atypical PKC inhibitor. Other studies also showed that leucine failed to stimulate Akt/PKB, 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 (47, 64). This led the authors to conclude that the downstream signal was different from that of insulin-stimulated glucose uptake. Furthermore, we have reported previously that BCAA-containing bioactive peptides, identified in whey protein hydrolysates, increased glucose uptake via the PI3-kinase and atypical PKC pathways in both L6 myotubes and isolated muscles (55). These findings
therefore indicate that certain amino acids and peptides may act directly to activate PKCζ.

Taken together, these results suggest that activation of Akt/PKB is related to insulin secretion, while activation of atypical PKC involves both amino acids and insulin action.

In this study we also confirmed a significant positive correlation between phosphorylated Akt/PKB, phosphorylated PKCζ, and skeletal muscle glycogen content. However, it has become increasingly clear that both Akt/PKB and atypical PKC are important for mediating the glucose transport effects of insulin, while Akt/PKB, rather than atypical PKC, appears to be important for stimulating glycogen synthesis and promoting glucose storage in muscle. Therefore, co-ingestion of glucose and whey protein hydrolysates may regulate glucose uptake primarily by activating Akt/PKB and atypical PKC, resulting in stimulation of glycogen synthesis by Akt/PKB.

In conclusion, our results demonstrate that ingestion of carbohydrate plus whey protein hydrolysates was more effective for increasing post-exercise skeletal muscle glycogen content in rats than ingestion of other protein sources. Post-exercise supplementation with carbohydrate and whey protein hydrolysates may function to increase skeletal muscle glycogen recovery due to activating Akt/PKB and atypical PKC, two key enzymes involved in the regulation of glucose uptake.
### Table 3-1. Amino acid composition of test diets (g/100 g protein).

<table>
<thead>
<tr>
<th></th>
<th>Whey protein and whey protein hydrolysates</th>
<th>Casein hydrolysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>4.78</td>
<td>3.05</td>
</tr>
<tr>
<td>Arg</td>
<td>2.95</td>
<td>3.46</td>
</tr>
<tr>
<td>Asx</td>
<td>10.52</td>
<td>6.37</td>
</tr>
<tr>
<td>Cys</td>
<td>2.55</td>
<td>0.13</td>
</tr>
<tr>
<td>Glu</td>
<td>16.81</td>
<td>21.08</td>
</tr>
<tr>
<td>Gly</td>
<td>1.92</td>
<td>1.79</td>
</tr>
<tr>
<td>His</td>
<td>2.19</td>
<td>2.99</td>
</tr>
<tr>
<td>Ile</td>
<td>5.40</td>
<td>4.57</td>
</tr>
<tr>
<td>Leu</td>
<td>11.57</td>
<td>9.47</td>
</tr>
<tr>
<td>Lys</td>
<td>9.44</td>
<td>7.31</td>
</tr>
<tr>
<td>Met</td>
<td>2.03</td>
<td>2.96</td>
</tr>
<tr>
<td>Phe</td>
<td>3.61</td>
<td>4.52</td>
</tr>
<tr>
<td>Pro</td>
<td>5.30</td>
<td>10.56</td>
</tr>
<tr>
<td>Ser</td>
<td>4.92</td>
<td>5.40</td>
</tr>
<tr>
<td>Thr</td>
<td>5.22</td>
<td>3.98</td>
</tr>
<tr>
<td>Trp</td>
<td>1.90</td>
<td>1.00</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.49</td>
<td>5.02</td>
</tr>
<tr>
<td>Val</td>
<td>5.37</td>
<td>6.32</td>
</tr>
<tr>
<td>IAA</td>
<td>46.73</td>
<td>43.13</td>
</tr>
<tr>
<td>BCAA</td>
<td>22.34</td>
<td>20.36</td>
</tr>
</tbody>
</table>

Indispensable amino acids (IAA): Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, His
Branched-chain amino acids (BCAA): Val, Leu, Ile
Figure 3-1. The experimental protocol.
**Figure 3-2.** Skeletal muscle glycogen content immediately after exercise and after 120 min of the recovery period, grouped according to the different type of test drinks ingested (n = 8/group). Values are means ± SEM. Mean values within a column with unlike superscript letters were significantly different (P < 0.05).
Figure 3-3. Phosphorylated a) Akt/PKB and b) PKCζ immediately after exercise and after 120 min of the recovery period, with ingestion of either glucose or glucose plus whey protein hydrolysates (n = 8/group). Values are means ± SEM. Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).
Figure 3-4. Correlation between skeletal muscle glycogen contents, and phosphorylated a) Akt/PKB and b) PKC$\zeta$. 
Chapter IV

Study 3

Branched-chain amino acid-containing dipeptides, identified from whey protein hydrolysates, stimulate glucose uptake rate in L6 myotubes and isolated skeletal muscles.
4.1. Introduction

It is well known that low- or moderate-intensity exercise has an insulin-like effect on glucose uptake in skeletal muscles (65, 66). In the absence of insulin, *in vitro* twitch or tetanic contractions by electrical stimulation cause an increase in muscle glucose uptake (67, 68). Furthermore, exercise training improves glucose tolerance and insulin action in subjects with either insulin-resistance or Type 2 diabetes (69-71). 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 (72). Although it is well established that exercise-training is an effective means of maximizing glucose uptake in skeletal muscles (73), 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 (74-77). 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 (78). We have also shown dietary whey protein, which is the main source of protein in dietary supplements, increased glycogen content in liver (76) and skeletal muscles in rats (75). However, it remains unclear whether ingredients of whey protein stimulate accumulation of glycogen in skeletal muscles.
Approximately 30 years 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 (79, 80). Recently, the intestinal oligopeptide transporter “Pept-1” was cloned in the intestines of human and experimental animals (81, 82). This led to the demonstration that di- and tri-peptides are actively and rapidly taken up by enterocytes via Pept-1. Flotz et al. (83) also reported that certain tripeptides 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.

4.2. Materials and Methods

4.2.1. 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 hr at 50°C with protease enzymes (1:100, enzyme –to-substrate ratio, w/w) from S. Bacillus and O. Aspergillus (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.

4.2.2. 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, Milford, MA, USA). The mobile phase A consisted of 0.05% trifluoro acetic acid (TFA) in Mill-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.

4.2.3. 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, Leu-Ile, Ile-Leu, Leu-Leu, and (231.1 > 86.1) for Ile-Val,
Leu-Val, Val-Leu. The parameters for the LC/MS/MS analysis of BCAA-containing dipeptides were as follows: capillary voltage, 3000 V; source temperature, 120°C; desolvation temperature, 400°C; desolvation gas flow, 849 L/hr; cone gas flow, 48 L/hr; cone voltage and collision energy set at 25 V and 15 V, respectively.

4.2.4. L6 myotubes cell culture

L6 myotubes (JCRB9081) 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 days. Cells were then cultured on 48-well culture plates (BD BioCoat™ Collagen coat I, Becton, Dickinson and Company, NJ, USA).

4.2.5. Glucose uptake assay in L6 myotubes

Glucose uptake was determined as described previously (84). 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 KRPH buffer containing 1 mM Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-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) (85) or 6 µM GF109203X, a specific inhibitor of atypical protein kinase C (aPKC) (86) in KRPH containing 1 mM Ile-Leu.

Glucose uptake was measured by adding 1 mM 2-deoxyglucose in KRPH 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. The LY294002, GF109203X, and cytochalasin B concentrations used were those which had resulted in inhibition in earlier cell experiments (78). Assay validity was checked by using 100 nM insulin (positive control). In addition, we confirmed that BCAA-containing dipeptides were stable (100 ± 5%) in sample buffer solution during the assay of 2-DG uptake.
4.2.6. 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-hr-light/-dark cycle. The study was approved by the Animal Committee of Meiji Seika Kaisha Ltd, Food and Health R&D Laboratories, with the animals receiving care under the guidelines laid down by this committee.

After 18 hrs starvation, the epitrochlearis muscles were dissected under sodium pentobarbital anesthesia (40 mg kg/BW i.p) (87). 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, 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 muscles were washed for 10 min in KHB containing 40 mM mannitol, 0.1% BSA, and either 1 mM Ile-Leu, 10 µM LY294002 or 6 µM GF109203X if that compound had been present in the earlier incubation. After being rinsed the muscles were incubated at 30°C for 20 min in 3 mL of KHB containing 8 mM 2-deoxyglucose (2DG), 32 mM mannitol, 0.1% BSA, in the presence or absence of 1 mM Ile-Leu, 10 µM LY294002, or 6 µM GF109203X. 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 weighted, homogenized in 0.3 M perchloric acid, and centrifuged at 3000 g for
10 min. After centrifuging, the neutralized supernatant was collected for the measurement of 2-deoxyglucose 6-phosphate. Validity check 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 during the assay.

4.2.7. Measurement of 2-DG uptake

Determination of 2-deoxyglucose 6-phosphate (2DG6P) was carried out according to the method of Ueyama et al. (88). Under the above conditions, intracellular accumulation of free 2DG in muscles is negligible, while intracellular accumulation of 2DG6P is linear. The intracellular accumulation rate of 2DG6P therefore reflects muscle glucose transport activity (89).

4.2.8. Glycogen contents in epitrochlearis muscles

Epitrochlearis muscles were placed in 3 mL of oxygenated KHB buffer containing 5 mM glucose, 35 mM mannitol, 0.1% BSA, 0.1% bovine serum albumin, with or without 1 mM Ile-leu (90). The muscles were incubated with shaking at 35°C, and the flasks were gassed continuously with 95% O₂, 5% CO₂. During 3 hr incubations, the muscles were placed in fresh incubation medium after 1.5 hr. After the incubation, muscles were used for measurement of glycogen levels. Glycogen was measured on perchloric acid homogenates of muscle using the
4.2.9. **Statistics**

Data were expressed as means ± SEM. The significance of the difference between the means of the test and control groups was established by either Student’s *t* test or one-way ANOVA with *post hoc* analyses being carried out by the Dunnett’s test or Tukey’s test. Differences between groups were considered to be statistically significant at *P* < 0.05.

4.3. **Results**

4.3.1. **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-Ile, 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.
4.3.2. 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 Figure 4-1, 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 4-2). Stimulation of glucose uptake in L6 cells by dipeptides was completely inhibited by treatment with either cytochalasin B (data not shown).

4.3.3. 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 (Figure 4-2). 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 (Figure 4-3).
4.3.4. Effect of Ile-Leu on glycogen contents in isolated muscles

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

4.4. 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-ketoisocaproic acid, a metabolite of leucine, promoted glucose uptake (78). 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 (54). 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.
uptake in the muscles.

In this study we used two different experimental systems to measure 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. (92) 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 muscle, although increased only twofold in L6 myotubes (data not shown). It is therefore important to examine glucose uptake using both L6 cells and isolated skeletal muscle, 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 muscle. This raised the possibility that other BCAA-containing dipeptides may also stimulate glucose uptake 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) (51) and aPKCs (PKC \( \lambda \) and \( \zeta \)) (93, 94). 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 GLUT-4 translocation (95). In this study, an increase in glucose uptake by 1mM 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. (78) 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 (47, 64). These authors therefore considered that the downstream signal was 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 GLUT-4 translocation by insulin (Figure 4-5).

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 (75, 76). This finding indicates that the type of dietary protein alters glucose metabolism in both skeletal muscle 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 5-1, β-lactoglobulin, the main component of whey protein, have numerous amino acid sequences of BCAA-containing dipeptides compared to other protein sources (96-98). Recently, Foltz et al. (83) reported that dietary tripeptides are absorbed intact into the circulation in human. 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 muscle. 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. (54, 60) 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. (48) 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 (47). 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 muscle possibly via the PI3-kinase and aPKC pathways, resulting in increased skeletal muscle glycogen contents.
Table 4-1. 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>
</tr>
</thead>
<tbody>
<tr>
<td>231.16</td>
<td>Val-Leu</td>
<td>β-Lactoglobulin</td>
<td>92-93</td>
</tr>
<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>α-Lactalbumin</td>
<td>60-61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-Lactoglobulin</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>
<tr>
<td></td>
<td>Ile-Ile</td>
<td>β-Lactoglobulin</td>
<td>71-72</td>
</tr>
<tr>
<td></td>
<td>Leu-Leu</td>
<td>β-Lactoglobulin</td>
<td>31-32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>57-58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>103-104</td>
</tr>
</tbody>
</table>
**Table 4-2.** Effect of BCAA-containing dipeptides on the rate of 2-deoxyglucose uptake in L6 myotubes. [nmol/10 min/well]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate (nmol/10 min/well) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>100 nM insulin (positive control)</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-Ile</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-Val</td>
<td>1.74 ± 0.06 (* )</td>
</tr>
<tr>
<td>1 mM Ile-Val</td>
<td>1.80 ± 0.07 (* )</td>
</tr>
</tbody>
</table>

Values are means ± SEM. (n = 8)

* P < 0.05 vs. control; Dunnett’s test
Figure 4-1. 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 ± SEM. (n = 8) * P < 0.05 vs. control (no addition, 0 mM); Dunnett’s test
Figure 4-2. 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 ± SEM (n = 8). Means in a column with superscripts without a common letter differ, P < 0.05; Tukey’s test.
Figure 4-3. Effect of 1 mM Ile-Leu, 10 µM LY294002, or 6 µM GF109203X on the rate of 2-deoxyglucose uptake in isolated epitrochlearis muscle. Values are means ± SEM (n = 8). Means in a column with superscripts without a common letter differ, $P < 0.05$; Tukey’s test.
Figure 4-4. Effect of 1 mM Ile-Leu on glycogen contents in isolated epitrochlearis muscle. Values are means ± SEM (n = 7). * P < 0.05 vs. control (no addition); Student’s t test.
**Figure 4-5.** Mechanism of GLUT-4 translocation induced by BCAA-containing peptides via insulin signaling pathway.
Chapter V

Study 4

Effect of post-exercise co-ingestion of carbohydrate plus whey protein hydrolysates on blood biochemical parameters of amino acid and carbohydrate metabolism, and exercise performance in trained men.
5.1. Introduction

In a recent study we showed post-exercise co-ingestion of glucose and whey protein hydrolysates compared to either glucose only, glucose plus casein hydrolysates, whey protein or branched-chain amino acids (BCAA), increased muscle glycogen repletion in exercise-trained rats (99). We have also demonstrated that BCAA-containing bioactive dipeptides isolated from whey protein hydrolysates stimulated insulin-independent glucose uptake and resulted in increased glycogen content in skeletal muscles (55). On the basis of this evidence we hypothesized that addition of whey protein hydrolysates (WPH) to carbohydrate drinks may improve skeletal muscle carbohydrate metabolism.

Increased amino acid oxidation during exercise is thought to be due to increased utilization of amino acids as fuel; therefore, regularly performed exercise would then lead to increased protein requirements (100). We speculate that the different amount of intake of dietary protein after exercise may have a different response to plasma amino acid availability and the insulin secretion in exercise-trained athletes. The aim of the present study was therefore to compare the effect of ingestion of carbohydrate alone and with two doses of whey protein hydrolysates (6.0 or 16.0 g/hr) on exercise performance and blood biochemical parameters of amino acid and carbohydrate metabolism during the post-exercise phase in trained men.
5.2. Materials and Methods

5.2.1. Subjects

Fifteen trained men were recruited to participate in this study. The protocol and potential benefits and risks associated with participation in the study were explained fully to each subject before they gave the informed consent document. The study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committee of the Faculty of Sport Sciences, Waseda University. Written informed consent was obtained from all subjects.

5.2.2. Preliminary testing

One week before the screening trial, the subjects performed a graded exercise test on an electromagnetically braked cycle ergometer (Combi Corporation, Tokyo, Japan) in order to determine maximal oxygen uptake (VO₂ max). The protocol consisted of a 4-min warm-up, followed by a series of 3-min stages, beginning at 90 W with workload increasing by 30 W at each stage until exhaustion. Breath-by-breath measurements were performed throughout the exercise session using an automated gas analysis system (AE280 Minato Medical Science, Osaka, Japan). The averages of inspired and expired volume, O₂ consumption (VO₂) and CO₂ production (VCO₂) were determined every 30 seconds.
5.2.3. Screening test

All subjects had previously been involved in cycle ergometry studies carried out under similar conditions to the present study, and were therefore fully familiar with the experimental procedures. Seven subjects were subsequently excluded as they were unable to complete the exercise protocol described below.

5.2.4. Experimental protocol

Eight trained men were selected to participate in the double-blind cross-over study. Their mean (± SEM) age, weight and VO\textsubscript{2} max were 22 ± 1 y, 61.1 ± 2.0 kg and 60.1 ± 3.1 ml\cdot kg/body wt/min, respectively.

To minimize differences in starting conditions, all the subjects were instructed to eat the same meals the day before the test. The diet intake in the 24-hr period before each time trial was 8700 kJ/d. In the hour preceding the tests, the subjects were not allowed to eat with the exception of water.

The experimental protocol was shown in Figure 5-1. The subjects assembled in a temperature-controlled room and then consumed a light breakfast 90 min before the test. The 70 min of cycling test consisted of six alternating work periods of 8 min at 68% VO\textsubscript{2} max and 2 min at 88% VO\textsubscript{2} max. The subjects cycled at 60% VO\textsubscript{2} max during the last 10 min. This protocol has been shown to result in very low muscle glycogen concentrations (101). During
the exercise tests, the subjects drank 100 mL of water every 20 min. Immediately after exercise and 30, 60, 90 and 120 min later, the subjects received a supplement containing either 1) CHO, 2) CHO plus a low amount (6.0 g/hr) of WPH (CHO + L-WPH), or 3) CHO plus a high amount (16.0 g/hr) of WPH (CHO + H-WPH). The composition of the test drinks are described below. After a 2-hr recovery period, an exercise performance test (time trial) was performed. Blood samples were drawn before exercise and at 0, 30, 60, 90 and 120 min after exercise, and also after the exercise performance test. All the trials were performed in mild environmental conditions (20–22°C) with an electric fan circulating air to minimize thermal stress.

5.2.5. Composition of test drink

During post-exercise recovery, the subjects received drinks (350 mL) containing either 17.5 g carbohydrate (CHO), 17.5 g carbohydrate plus 3 g whey protein hydrolysates (CHO + L-WPH), or 17.5 g carbohydrate plus 8 g whey protein hydrolysates containing drink (CHO + H-WPH) at 0, 30, 60, 90 min. The beverages were flavored to make the taste comparable in all trials. The amount of carbohydrate (17.5 g) was calculated from a commercial sports drink (5%, 350 mL). The amount of WPH (3 g) was added equal to the amount of commercial amino acids supplement, while the amount of WPH (8 g) was equivalent to the protein content of a glass of milk (8 g/250 mL). Carbohydrates were provided as maltodextrin
(Matsutani Chemical Industries, Co., Ltd.). Whey protein hydrolysates (Meiji Seika Kaisha Ltd., Tokyo, Japan) were prepared by enzymatic hydrolysis (Morifuji et al, 2009a) and had an average peptide length of 3.64, calculated as the TN ratio (total nitrogen)/AN (amino nitrogen). The whey protein hydrolysates contained BCAA-containing bioactive peptides, including 0.92 mg Ile-Val, 0.87 mg Leu-Val, 7.02 mg Val-Leu, 0.33 mg Ile-Ile, 0.06 mg Leu-Ile, 4.57 mg Ile-Leu and 7.86 mg Leu-Leu per gram of sample. The amino acid (AA) profile of the whey protein was as follows; arginine 2.8%, lysine 9.8%, histidine 2.2%, phenylalanine 3.3%, tyrosine 3.5%, leucine 11.1%, isoleucine 4.8%, methionine 2.2%, valine 5.0%, alanine 4.9%, glycine 1.9%, proline 5.5%, glutamic acid (glutamine) 17.7%, serine 5.0%, threonine 5.3%, aspartic acid (asparagine) 10.9%, tryptophan 1.8% and cystine 2.4%.

5.2.6. Protocol of exercise performance test (Time trial)

The time trial tests were carried out using a cycle ergometer with a constant work load. Subjects were motivated to perform a set amount of work as fast as possible. To avoid test-retest influence, the subjects received no information about the time, heart rate or pedaling rate and were only aware of the work performed.

Work load and the amount of work (kJ) were calculated as:
\[
\text{Work load ( kpm/min ) } = \frac{60\% \text{ VO}_2 \text{ max (W)}}{0.163 \times 6 \times 70 \text{ (rpm)}}
\]

\[
\text{The amount of work ( kJ ) } = \frac{60\% \text{ VO}_2 \text{ max (W)} \times 0.0023 \times 40 \text{ (min)}}{0.163 \times 0.232 \times 0.239 \text{ (J)}}
\]

5.2.7. Blood analyses

Blood was collected into plain tubes for free fatty acids (FFA), EDTA-containing tubes for insulin, hematological parameters (hematocrit, hemoglobin, red and white blood cell counts), amino acids, peptides and cytokines, and EDTA plus sodium fluoride-containing tubes for glucose. After collection, the blood samples were centrifuged at 1000 g at 4°C for 15 min, and the serum and plasma samples stored at -80°C until assayed. All these variables, with the exception of amino acids, peptides and cytokines, were assayed using standard laboratory techniques (BML Inc, Tokyo, Japan).

Plasma free amino acids were determined by HPLC, with precolumn 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization (102) and BCAA-containing dipeptides identified using LC/MS/MS systems (Quatro premier XE, Waters Corporation, Milford, MA, USA).

The levels of IL-6, IL-10 and IL-1ra were assayed using ELISA kits (IL-6 and IL-1ra,
5.2.8. **Statistical analysis**

Data are expressed as means ± SEM. Blood glucose and plasma insulin, amino acids and dipeptide responses were calculated as area under the curve minus baseline values (AUC). The effect of each treatment was compared with that of the control trial using paired *t* tests. Differences between groups were considered significant at *P* < 0.05. Associations between the variables were examined using Pearson’s correlation coefficient.

5.3. **Results**

5.3.1. **Blood glucose and insulin**

Figure 5-2 shows the changes in blood glucose and plasma insulin concentrations over the study period. Blood glucose levels were significantly lower 90 and 120 min after ingestion of CHO + H-WPH, compared to CHO only. When the data were expressed as AUC for the entire 120 min of the trial, blood glucose levels were confirmed to be lower following co-ingestion of CHO + H-WPH, compared to CHO only.

There was also a significant increase in plasma insulin at 30, 60, 90 and 120 min after ingestion of CHO + H-WPH compared with ingestion of CHO only. Data expressed as AUC for the entire 120 min trial showed plasma insulin levels were also significantly higher after
administration of CHO + H-WPH, compared to administration of CHO only.

### 5.3.2. Plasma concentrations of amino acids and dipeptides

Figure 5-3 shows the changes in concentrations of plasma total amino acids (TAA), indispensable amino acids (IAA) and branched chain amino acids (BCAA). Beverages containing CHO + L-WPH caused significant increases in plasma TAA, IAA and BCAA levels at 30, 60, 90 and 120 min compared to the CHO beverage. In the CHO + H-WPH group, plasma TAA, IAA, BCAA levels after the performance test were also significantly increased, compared to the CHO group. Data expressed as AUC for the entire 120 min trial confirmed that the concentrations of plasma TAA, IAA and BCAA were higher in the CHO + L-WPH and CHO + H-WPH groups, compared to the CHO group.

Table 5-1 shows the total area under the curve for plasma amino acids and dipeptides for the recovery period (0-120 min) after exercise. The CHO + H-WPH drink increased plasma amino acids significantly, with the exception of aspartic acid and glutamic acid. Similarly, the CHO + L-WPH drink increased amino acids significantly compared to the CHO drink, except for aspartic acid, cystine, glycine and glutamic acid.

Table 5-2 shows the correlation coefficients and $P$ values for the relationship between plasma amino acids, dipeptides and insulin. There was a significant positive correlation between the plasma concentrations of insulin and amino acids except for alanine, aspartic acid.
and glutamic acid ($P < 0.05$). In addition, a significant positive correlation was observed between the plasma concentrations of insulin and the dipeptides, Ile-Val, Val-Leu, Ile-Leu and Leu-Leu ($P < 0.05$).

5.3.3. Other blood parameters

As shown in Figure 5-4, we also observed a significant decrease in serum free fatty acids (FFA) at 30 and 120 min following ingestion of CHO + H-WPH, compared with ingestion of CHO only. Serum FFA and plasma IL-6 levels after the performance test were significantly lower in the CHO + H-WPH group than in the CHO group. However, plasma IL-10, IL-1ra, white and red blood cell counts, hematocrit and hemoglobin levels were not different between the groups (data not shown).

5.3.4. Exercise performance test

Table 5-3 shows the results of the exercise performance test. Endurance performance was not statistically different between the three groups.

5.4. Discussion

This study demonstrated co-ingestion of CHO + H-WPH was more effective than ingestion of CHO alone for stimulating insulin secretion and plasma amino acid and dipeptide
availability in exercise-trained athletes. Furthermore, serum FFA and plasma IL-6 levels after
the performance test were significantly lower in the CHO + H-WPH group than in the CHO
group.

It is well established that insulin is an important factor in carbohydrate metabolism.
Insulin stimulates muscle glucose utilization by activating glucose transport, a process
generally considered to be the major factor determining the rate of glycogen synthesis when
substrate supply is adequate (12-14). This study demonstrated that the plasma insulin response
was increased significantly in the CHO + H-WPH (\(0.58 \text{ g/kg/hr (CHO) + 0.26 g/kg/hr (WPH)}\)) group compared to the CHO (\(0.58 \text{ g/kg/hr}\)) group, but not the CHO + L-WPH (\(0.58 \text{ g/kg/hr (CHO) + 0.10 g/kg/hr (WPH)}\)) group. Plasma concentrations of amino acids
during the recovery period were increased markedly following ingestion of a high dose of
WPH, whereas they increased only marginally with a lower dose of WPH, due to plasma
amino acid levels in the CHO group decreasing compared with levels seen immediately after
exercise. Low plasma concentrations of amino acids in the L-WPH group may therefore
diminish the response of insulin secretion. Taken together these results indicate insulin
responses may be related to postprandial plasma amino acid concentrations.

The synergistic stimulating effect of combined ingestion of carbohydrate and intact
protein on plasma insulin release was first reported in the late 1960s and was later confirmed
in both healthy subjects (17, 18) and patients with type 2 diabetes (15, 16). Several \textit{in vitro}
studies, using incubated β pancreatic cells, also showed arginine, isoleucine, phenylalanine and leucine in combination with glutamine had a strong insulinotropic effect (103, 104). In addition, Nilsson et al. (62) reported a close relationship between the insulin response and increase in plasma amino acid levels in humans. This relationship was especially apparent for leucine, isoleucine, valine, lysine and threonine following ingestion of whey protein. Our study also provides evidence that whey protein causes marked increases in the plasma concentrations of certain amino acids, especially isoleucine, leucine, threonine, lysine and valine. We also found significant, strong positive correlations between the levels of plasma insulin and several amino acids, including isoleucine, leucine, threonine, tryptophan, phenylalanine and methionine. These amino acids may be associated with an increase in insulin secretion. In addition, we also observed a significant, positive correlation between the levels of plasma insulin and Val-Leu and Ile-Leu. However, the mechanism by which these BCAA-containing dipeptides exert this effect is not fully understood.

This study showed blood glucose levels were decreased significantly in the CHO + H-WPH group, compared to the CHO group, a finding attributable to the stimulating effect of insulin on skeletal muscle glucose uptake. Both glucose availability and insulin concentrations determine the rate of glucose uptake in skeletal muscle, with insulin-stimulating glucose utilization in muscle cells by activating glucose transport (GLUT-4 translocation). Furthermore, there is evidence from a recent study that BCAAs, such as
leucine and isoleucine, and BCAA-containing bioactive peptides increase insulin-independent glucose uptake activity in skeletal muscles (54, 55, 78). WPH contains large amounts of BCAA and BCAA-containing bioactive peptides, and in our study we detected these types of peptides, mainly Ile-Val, Val-Leu, Ile-Leu in the plasma. As insulin-independent glucose uptake may also be related to suppression of blood glucose levels, it is possible that increased plasma levels of certain amino acids or bioactive peptides may induce a larger increase in glucose uptake by both insulin-dependent and insulin-independent mechanisms, thereby resulting in decreased blood glucose levels.

Several studies have reported that post exercise muscle glycogen storage can be facilitated when protein is added to lower quantities of carbohydrate (< 0.8 g/kg/hr). In fact, recent evidence from muscle biopsies indicates that ingesting protein after exercise may have a beneficial effect on muscle glycogen storage. For example, studies have shown that a combination of carbohydrate and protein was more effective than carbohydrate alone for replenishing muscle glycogen during the 4 hr period immediately after exercise (32, 77). This effect was also demonstrated by van Loon et al. (3). Ivy et al. (4) observed a rapid increase in muscle glycogen storage during the first 40 min of recovery with combined carbohydrate and protein treatment. These beneficial effects of glycogen accumulation during the post-exercise recovery were accompanied by changes in parameters of carbohydrate metabolism, such as a greater plasma insulin response and/or lower blood glucose response. The current study also
demonstrated that plasma insulin levels were higher in the H-WPH group than in the CHO group, while blood glucose levels were lower. Taken together, these results may indicate co-ingestion of CHO + H-WPH stimulates muscle glycogen synthesis during the post-exercise recovery state.

This study showed that IL-6 levels were significantly lower in the CHO + H-WPH group after exercise performance test, although white blood cell count and plasma IL-10 and IL-1ra levels were not different between the treatment groups. In addition, co-ingestion of CHO + H-WPH, compared to CHO only, lowered serum FFA levels after the exercise performance test. Several studies have reported that carbohydrate ingestion attenuates elevations in plasma IL-6 during both running and cycling (105, 106). During exercise, carbohydrate ingestion exerts its effect at the posttranscriptional level of IL-6 (107, 108), whereas low muscle glycogen concentration further enhances IL-6 mRNA expression and transcription rate (109, 110). Therefore, pre-exercise intramuscular glycogen content appears to be an important stimulus for IL-6 gene transcription, and it appears that muscle-derived IL-6 acts as an energy sensor (111). It is also known that high glycogen status, compared to low glycogen, attenuates the increases in serum FFA that occur during exercise (112). There is evidence that co-ingestion of carbohydrate plus protein enhances carbohydrate oxidation and decreases utilization of serum FFA (113). These results therefore suggest that the larger increase in muscle glycogen during the recovery period was utilized preferentially as the main energy...
source during the exercise performance tests, thereby resulting in suppression of plasma IL-6 and serum FFA in the CHO + H-WPH group.

In this study, endurance performance was not statistically different between the three groups. While the effect of carbohydrate-protein ingestion on endurance performance remains controversial, the observation of a prolonged time to exhaustion in the studies by Betts et al. (114) and Williams et al. (115) suggests that carbohydrate-protein beverages may have significant ergogenic effects. However, as demonstrated by Van Essen and Gibala (116), improvements in endurance performance have not been observed universally with carbohydrate-protein ingestion. Further studies are therefore needed to clarify the conditions under which the presence of protein in a sports beverage may improve performance.

In conclusion, this study demonstrated that co-ingestion of CHO + H-WPH was more effective than ingestion of CHO alone for increasing insulin secretion, plasma amino acid levels and dipeptide availability, and decreasing blood glucose response in exercise-trained athletes. Furthermore, blood parameters of substrate metabolism, such as FFA and IL-6, also changed after co-ingestion of CHO + H-WPH. These changes in blood parameters therefore support the concept that ingestion of CHO + WPH has a beneficial role of increasing post-exercise glycogen repletion rates.
Figure 5-1. Experimental protocol
**Figure 5-2.** A) Blood glucose and B) plasma insulin levels. The left-hand graph shows the concentrations of blood glucose and plasma insulin over the study period, while the right-hand graph shows the area under the curve (AUC) for the 0-120 min recovery period. Values are means ± SEM, n = 8/group. * P < 0.05 significant difference vs the carbohydrate group.
Figure 5-3. Plasma concentrations of A) total amino acids, B) indispensable amino acids, and C) branched-chain amino acids. The left-hand graph shows the plasma concentrations of these amino acid groups over the study period, while the right-hand graph shows the area under the curve (AUC) for the 0-120 min recovery period. Values are means ± SEM, n = 8/group. * P < 0.05 significant difference vs the carbohydrate group.
Figure 5-4. A) Plasma IL-6 and B) serum free fatty acids levels over the study period. Values are means ± SEM, n = 8/group. * P < 0.05 significant difference vs carbohydrate group.
Table 5-1. Total area under the curve for plasma amino acids and dipeptides for the 0-120 min period after exercise

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th>CHO + L-WPH</th>
<th>CHO + H-WPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µmol · min/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>-12007 ± 2384</td>
<td>-7085 ± 933 *</td>
<td>2032 ± 1253 *</td>
</tr>
<tr>
<td>Arg</td>
<td>-628 ± 684</td>
<td>1353 ± 570 *</td>
<td>5748 ± 536 *</td>
</tr>
<tr>
<td>Asn</td>
<td>-563 ± 184</td>
<td>896 ± 66 *</td>
<td>3730 ± 185 *</td>
</tr>
<tr>
<td>Asp</td>
<td>TRA</td>
<td>TRA</td>
<td>TRA</td>
</tr>
<tr>
<td>Cys</td>
<td>-338 ± 173</td>
<td>331 ± 285</td>
<td>1343 ± 319 *</td>
</tr>
<tr>
<td>Gln</td>
<td>-4944 ± 2459</td>
<td>1573 ± 933 *</td>
<td>9119 ± 1144 *</td>
</tr>
<tr>
<td>Glu</td>
<td>-1614 ± 194</td>
<td>-2002 ± 216 *</td>
<td>-890 ± 324</td>
</tr>
<tr>
<td>Gly</td>
<td>-1813 ± 434</td>
<td>-1127 ± 249</td>
<td>1253 ± 759 *</td>
</tr>
<tr>
<td>His</td>
<td>-384 ± 161</td>
<td>572 ± 189 *</td>
<td>1799 ± 325 *</td>
</tr>
<tr>
<td>Ile</td>
<td>-1142 ± 79</td>
<td>2457 ± 177 *</td>
<td>10988 ± 437 *</td>
</tr>
<tr>
<td>Leu</td>
<td>-2355 ± 263</td>
<td>6641 ± 454 *</td>
<td>25817 ± 1047 *</td>
</tr>
<tr>
<td>Lys</td>
<td>-1499 ± 666</td>
<td>5077 ± 1961 *</td>
<td>19322 ± 1632 *</td>
</tr>
<tr>
<td>Met</td>
<td>-575 ± 72</td>
<td>174 ± 85 *</td>
<td>2468 ± 174 *</td>
</tr>
<tr>
<td>Phe</td>
<td>-935 ± 153</td>
<td>277 ± 147 *</td>
<td>2866 ± 180 *</td>
</tr>
<tr>
<td>Pro</td>
<td>-2185 ± 543</td>
<td>577 ± 253 *</td>
<td>7748 ± 979 *</td>
</tr>
<tr>
<td>Ser</td>
<td>-1685 ± 370</td>
<td>57 ± 283 *</td>
<td>4476 ± 565 *</td>
</tr>
<tr>
<td>Thr</td>
<td>-1885 ± 374</td>
<td>1463 ± 219 *</td>
<td>8756 ± 945 *</td>
</tr>
<tr>
<td>Trp</td>
<td>-489 ± 156</td>
<td>1441 ± 190 *</td>
<td>5093 ± 266 *</td>
</tr>
<tr>
<td>Tyr</td>
<td>-1034 ± 147</td>
<td>786 ± 178 *</td>
<td>5486 ± 379 *</td>
</tr>
<tr>
<td>Val</td>
<td>-2571 ± 360</td>
<td>3393 ± 615 *</td>
<td>17649 ± 857 *</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(nmol · min/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile-Val</td>
<td>-6.45 ± 18.03</td>
</tr>
<tr>
<td>Leu-Val</td>
<td>6.90 ± 19.59</td>
</tr>
<tr>
<td>Val-Leu</td>
<td>37.6 ± 40.7</td>
</tr>
<tr>
<td>Ile-Ile</td>
<td>-13.9 ± 28.1</td>
</tr>
<tr>
<td>Ile-Leu</td>
<td>11.1 ± 42.8</td>
</tr>
<tr>
<td>Leu-Ile</td>
<td>-10.1 ± 14.8</td>
</tr>
<tr>
<td>Leu-Leu</td>
<td>-24.6 ± 32.4</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.
TRA: detected at trace level.
Table 5-2. Correlation coefficients and $P$ values for the relationship between plasma amino acids, dipeptides and insulin.

<table>
<thead>
<tr>
<th></th>
<th>$r$</th>
<th>$P$</th>
<th></th>
<th>$r$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>0.377</td>
<td>0.070</td>
<td>Leu</td>
<td>0.669</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Arg</td>
<td>0.405</td>
<td>0.049</td>
<td>Lys</td>
<td>0.453</td>
<td>0.026</td>
</tr>
<tr>
<td>Asn</td>
<td>0.606</td>
<td>0.002</td>
<td>Met</td>
<td>0.776</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Asp</td>
<td>-</td>
<td>-</td>
<td>Phe</td>
<td>0.700</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cys</td>
<td>0.408</td>
<td>0.048</td>
<td>Pro</td>
<td>0.612</td>
<td>0.001</td>
</tr>
<tr>
<td>Gln</td>
<td>0.512</td>
<td>0.011</td>
<td>Ser</td>
<td>0.606</td>
<td>0.002</td>
</tr>
<tr>
<td>Glu</td>
<td>0.173</td>
<td>0.420</td>
<td>Thr</td>
<td>0.676</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gly</td>
<td>0.419</td>
<td>0.042</td>
<td>Trp</td>
<td>0.693</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>His</td>
<td>0.420</td>
<td>0.041</td>
<td>Tyr</td>
<td>0.681</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ile</td>
<td>0.704</td>
<td>&lt;0.001</td>
<td>Val</td>
<td>0.588</td>
<td>0.002</td>
</tr>
<tr>
<td>TAA</td>
<td>0.732</td>
<td>&lt;0.001</td>
<td>BCAA</td>
<td>0.751</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EAA</td>
<td>0.746</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile-Val</td>
<td>0.627</td>
<td>0.001</td>
<td>Ile-Ile</td>
<td>0.395</td>
<td>0.056</td>
</tr>
<tr>
<td>Leu-Val</td>
<td>0.350</td>
<td>0.093</td>
<td>Leu-Ile</td>
<td>0.269</td>
<td>0.204</td>
</tr>
<tr>
<td>Val-Leu</td>
<td>0.870</td>
<td>&lt;0.001</td>
<td>Ile-Leu</td>
<td>0.789</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leu-Leu</td>
<td>0.580</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5-3. Results of the exercise performance test in the eight study participants.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>CHO</th>
<th>CHO + L-WPH (sec)</th>
<th>CHO + H-WPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2294</td>
<td>2283</td>
<td>1988</td>
</tr>
<tr>
<td>B</td>
<td>1854</td>
<td>1836</td>
<td>1892</td>
</tr>
<tr>
<td>C</td>
<td>1870</td>
<td>1816</td>
<td>1856</td>
</tr>
<tr>
<td>D</td>
<td>2673</td>
<td>2485</td>
<td>2096</td>
</tr>
<tr>
<td>E</td>
<td>2434</td>
<td>2957</td>
<td>2409</td>
</tr>
<tr>
<td>F</td>
<td>2514</td>
<td>2738</td>
<td>2183</td>
</tr>
<tr>
<td>G</td>
<td>1952</td>
<td>1888</td>
<td>2251</td>
</tr>
<tr>
<td>H</td>
<td>2080</td>
<td>2127</td>
<td>2077</td>
</tr>
</tbody>
</table>

| Mean        | 2209 | 2266              | 2094        |
| SEM         | 111  | 152               | 66          |
Chapter VI

Discussion and Conclusion
6.1. Discussion and Conclusion

This study firstly demonstrated that chronic ingestion of whey protein was more effective protein source for the accumulation of skeletal muscle glycogen content in rats (Study 1). Furthermore, post-exercise ingestion of whey protein hydrolysates increased muscle glycogen repletion in rats (Study 2). In human, postprandial plasma insulin levels were significantly higher after administration of carbohydrate plus whey protein hydrolysates, compared to administration of carbohydrate only, while blood glucose levels were also lower. These results may indicate co-ingestion of carbohydrate plus whey protein hydrolysates stimulates muscle glycogen synthesis during the post-exercise recovery state because effects of glycogen accumulation during the post-exercise recovery were accompanied by changes in parameters of carbohydrate metabolism, such as a greater plasma insulin response and lower blood glucose response (Study 4).

Thus, these results showed that daily intake of whey protein/protein hydrolysates as dietary supplements, and co-ingestion of whey protein/protein hydrolysates with commercial sports drink after exercise may enhance the storage of skeletal muscle glycogen. However, there is only limited information whether whey protein increases the storage of skeletal muscle glycogen in human. Further clinical studies are needed to clarify the effect on glycogen accumulation in skeletal muscle.
Table 6-1.

<table>
<thead>
<tr>
<th>Study</th>
<th>Rats</th>
<th>Chronic feeding</th>
<th>Skeletal muscle glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>for 14 days</td>
<td>Whey &gt; Casein</td>
</tr>
<tr>
<td>2</td>
<td>Rats</td>
<td>Single administration</td>
<td>Whey peptide &gt; Whey protein &gt; Casein peptide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-exercise phase</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>human</td>
<td>Single administration</td>
<td>Increase in plasma insulin and decrease in blood glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-exercise phase</td>
<td>Whey peptide + Carbohydrate &gt; Carbohydrate</td>
</tr>
</tbody>
</table>

Furthermore, these experiments showed that whey protein modulates skeletal muscle glycoregulatory enzyme activities, and phosphorylated signaling molecules, key enzymes regulating glucose uptake. It is possible that these changes may be activated by both insulin-dependent and insulin-independent mechanisms.

Insulin is a strong activator of muscle glycogen synthesis due to its stimulating effect on glucose transport and glycogen synthesis. Although pancreatic insulin secretion is regulated primarily by the concentration of blood glucose, protein as well as some amino acids also stimulates insulin secretion. Co-ingestion of glucose and whey protein hydrolysates stimulate insulin release to a greater extent than glucose alone, suggesting that this elevation in insulin level, may in turn, activate glucose transport and glycogen synthesis in skeletal muscle (study 4).

A recent study demonstrated that certain amino acids directly activate the key proteins in the insulin signaling pathway. Doi et al. (54, 60) 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. Furthermore, leucine stimulates glycogen synthesis as a result of the inactivation of glycogen synthase kinase-3 in L6 cells (47).

However, ingestion of whey protein hydrolysates caused significantly greater increases in skeletal muscle glycogen levels than non-hydrolysed whey protein even though their amino acid composition was the same (Study 2). These results therefore indicated that another factor, other than the amino acid composition of dietary protein, contributed to post-exercise glycogen repletion. We demonstrated in an *in vitro* study that BCAA-containing bioactive peptides such as Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Ile-Leu, Leu-Ile, and Leu-Leu increased the rate of glucose uptake, resulting in accumulation of glycogen (Study 3). Therefore, certain bioactive peptides, such as BCAA-containing peptides, may also contribute to enhanced post-exercise skeletal muscle glycogen levels. However, the manner in which these ingredients may exert this effect is not fully understood.

**Conclusion:** whey protein/protein hydrolysates were more effective for the accumulation of skeletal muscle glycogen content. However, further studies are needed to clarify active components of whey protein.
Figure 6-1. Mechanism of glycogen accumulation when ingested whey protein

Estimated mechanism of glycogen accumulation by whey protein/protein hydrolysates was summarized as follows;

- Ingested dietary protein was digested in gastrointestinal organs, and absorbed as amino acids and di- and tri-peptides.
- Plasma amino acids and dipeptides levels were elevated in the circulation (Study 4).
- Several amino acids stimulate insulin release from β pancreatic cells (Study 4).
- Insulin stimulated skeletal muscle glucose uptake, glycogen synthesis via insulin signaling pathway (Study 1- Study 3).
- Certain amino acids and dipeptides directly stimulated skeletal muscle glucose uptake, glycogen synthesis via insulin signaling pathway (Study 1- Study 3).
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Appendix

1. Dietary whey protein increases liver and skeletal muscle glycogen levels in exercise-trained rats.
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2. Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats.
   Morifuji M, Kanda A, Koga J, Kawanaka K, Higuchi M.
   *Amino Acids.* 2009 *in press*

3. Branched-chain amino acid-containing dipeptides, identified from whey protein hydrolysates, stimulate glucose uptake rate in L6 myotubes and isolated skeletal muscles.
   Morifuji M, Koga J, Kawanaka K, Higuchi M.