Dietary Whey Protein Modulates Liver Glycogen Level and Glycoregulatory Enzyme Activities in Exercise-Trained Rats

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This study compared the effects of dietary whey protein with dietary casein or soy protein on glycogen storage and glycoregulatory enzyme activities in the liver of sedentary and exercise-trained rats. Male Sprague-Dawley rats (ca. 130 g) were divided into one sedentary and three exercise-trained groups, with eight animals in each group. Casein was provided as the source of dietary protein in the sedentary group while the exercise-trained groups were fed casein, whey, or soy protein. Rats in the exercise-trained groups ran for 30 mins/day, 4 days/week on a motor-driven treadmill. In the exercise-trained rats, animals fed whey protein had higher liver glycogen content than animals in the other two diet groups. Glucokinase activity was significantly higher in rats fed whey protein compared to that in rats fed soy protein, while glucose 6-phosphatase activity was significantly decreased in animals on the whey protein diet compared with those the other two diets. Although 6-phosphofructokinase activity was significantly lower in the whey protein group than in the soy protein group, we found that fructose 1,6-bisphosphatase activity was significantly higher in the whey group compared with either the casein or soy groups. Pyruvate kinase activity in rats fed the casein diet was significantly higher than in rats fed either the whey or soy protein diets. In addition, hepatic alanine aminotransferase activity and serum alanine level were also increased in the whey protein group compared with the casein or soy protein groups. Taken together, these results demonstrate that the whey protein diet in exercise-trained rats results in significantly higher levels of liver glycogen, because of the combined effects of regulation of rate limiting glycolytic and gluconeogenic enzyme activities and activation of glycogenesis from alanine via alanine aminotransferase. Exp Biol Med 230:23–30, 2005

Key words: whey protein; liver glycogen; glycoregulatory enzymes; exercise-trained rats

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Introduction

During both sprint and prolonged endurance exercise, the body must carefully balance availability and utilization of fuel in liver and skeletal muscle. Between meals, liver glycogen is used to maintain the level of blood glucose, while during the postprandial period (i.e., the first 2–3 hours after feeding), dietary glucose is distributed within the body according to a set pattern. Approximately one-third is used by the brain, undergoing complete oxidation to CO_2 and H_2O; one-third is taken up by skeletal muscle, where it is either stored as glycogen or metabolized to lactate or CO_2 and H_2O, according to the intensity of muscle activity; and the majority of the remaining one-third is taken up by the liver, primarily to be stored as glycogen (1–3).

In the liver, several important pathways play a role in glucose homeostasis by maintaining a balance between glucose uptake and storage, determined by glucogenesis and glycolysis, and glucose release governed by glycogenolysis and gluconeogenesis. In addition to these pathways, amino acids are transaminated so that the carbon skeleton of these compounds can be used in the gluconeogenic or ketogenic pathways. There is evidence that protein degradation is increased by exercise, with the serum level of essential amino acids being shown to increase after a bout of exercise (4). Amino acids produced by the breakdown of proteins can be either used for synthesis of new proteins or used in the production of glucose or ketone bodies (5). Therefore, when investigating mechanisms that contribute to the accumulation of glycogen in the liver, it is important to examine the activities of regulatory enzymes involved in carbohydrate metabolism.

Many athletes use protein supplements to stimulate and maintain muscle growth and strength and also to stimulate the release of growth hormone. A review by Lemon (6) indicated that strength athletes should consume approximately 12%–15% of their daily total energy intake as protein, equivalent to about 1.5–2.0 g protein/kg per day. The main sources of protein in these supplements are casein, whey, or soy proteins. Whey protein comprises about 20% of the total bovine milk protein and consists mainly of α-

lactoglobulin (50%), β-lactalbumin (25%), serum albumin (7%) and immunoglobulins (5%). In nature, whey protein contains high concentrations of branched chain amino acids—compounds that in addition to being involved in protein synthesis (7) also have an important role in carbohydrate metabolism (8) by being an important source of energy during exercise.

Depletion of liver glycogen stores has been associated with fatigue during constant-load exercise (9–11), and it is known that the storage levels are reduced in patients with liver cirrhosis or following surgery (12). Maintaining adequate stores of tissue glycogen is therefore important in both clinical and sports-related situations. However, although it is well established that dietary carbohydrate is an effective source of tissue glycogen, few nutritional studies have been able to demonstrate that other dietary components, such as protein, have beneficial effects on this process. This study was designed with the aim of comparing the effects of different types of dietary protein on glycogen content and glycoregulatory enzyme activities in the liver of exercise-trained rats.

Materials and Methods

Animals. Male Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan), with an initial body weight of approximately 130 g were used in this experiment. All rats were individually housed in temperature-controlled rooms (22°C) with light from 0800 to 2000 hrs and dark from 2000 to 0800 hrs. The study was approved by the Animal Committee of Meiji Seika Kaisha Ltd., Health & Bioscience Laboratories, with the animals receiving care under the guidelines laid down by this committee.

Diets. The design of the experimental diets followed the AIN-93 protocol (13), with the composition of the diets shown in Table 1. Casein, whey protein, and soy protein were used as the source of dietary protein. The protein content, calculated as nitrogen concentration × 6.38 (casein, whey protein) or × 5.71 (soy protein), was measured using the Kjeldahl method. Casein (87.7 g crude protein/100 g), whey protein (84.0 g crude protein/100 g), and soy protein (77.2 g crude protein/100 g) were added as 200 g of protein per 1 kg to the diets. The difference in the protein content between the three diets was compensated for by the addition of corn starch.

Experimental Protocol. All the rats were allowed free access to food and water for 4 weeks and were then divided into one sedentary and three exercise-trained groups, with eight animals in each group. The sedentary group was fed casein as the source of dietary protein, and the three exercise-trained groups were fed casein, whey protein, or soy protein. Rats in the exercise-trained groups ran on a motor-driven treadmill (Muromachi Kikai Co., Ltd., Tokyo, Japan) for 30 mins/day, 4 days/week. The running speed was 20 m/min at a 7° inclination during the first 5 mins, and 25 m/min at the same inclination during the last 25 mins. At the end of the 4 weeks of training, 24 hrs after the end of the last training session, all the rats fasted for 12 hrs to eliminate any confounding effects of the last meal. The rats were sacrificed between 0900 and 1000 hrs. Arteriovenous blood was collected under ether anesthesia. The blood sample was then centrifuged at 3000 *g* for 15 min and the serum stored at –80°C. After blood collection, the abdominal cavity was opened and the liver quickly excised, washed, weighed, and frozen at –80°C for later analysis.

Table 1. Composition of the Three Protein Diets (g/kg Diet)

Manufacturer[a]		Casein	Whey protein	Soy protein
1	Casein	228	—	—
2	Whey protein	—	238	—
3	Soy protein	—	—	259
4	Vitamin mixture	10	10	10
5	Choline bitartrate	2.5	2.5	2.5
4	Mineral mixture	35	35	35
6	Corn oil	70	70	70
7	Corn starch	504.5	494.5	473.5
8	Sucrose	100	100	100
9	Cellulose	50	50	50

[a] 1, Oriental Yeast, Co., Ltd., Tokyo, Japan; 2, Nihon NZMP Co., Ltd., Tokyo, Japan; 3, Fuji Oil Co., Ltd., Osaka, Japan; 4, AIN-93 diet. Nosan Corporation, Kanagawa, Japan; 5, Wako Pure Chemical Industries, Ltd., Osaka, Japan; 6, Ajinomoto Co., Inc., Tokyo, Japan; 7, Taiyo Kagaku Co., Ltd., Mie, Japan; 8, Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan; 9, Asahi Kasei Corporation, Tokyo, Japan.

Serum Glucose, Insulin, and Glucagon Analyses. Serum glucose concentration was measured using a glucose oxidase assay (14), serum insulin concentration by an enzyme-linked immunosorbent assay (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.

Glycogen Analysis. Tissue glycogen was isolated and purified by precipitation with ethanol from a digest formed by the addition of a 300 g/l potassium hydroxide solution and then quantified by the phenol-sulfuric acid method (15).

Enzyme Activities. Aliquots of liver were homogenized in 0.1 *M* Tris-HCl buffer (pH 7.4) using a Potter-Elvehjem type homogenizer fitted with a Teflon pestle. The homogenate was centrifuged for 30 mins at 12,000 *g* at 4°C and the supernant used immediately to determine the enzyme activities.

Glucokinase (EC 2.7.1.1), 6-phosphofructokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40), phosphoenolpyruvate carboxykinase (EC 4.1.1.32), and fructose 1,6-bisphosphatase (EC 3.1.3.11) were assayed spectrophotometrically. Glucokinase was assayed according to Grossman *et al.* (16). The assay mixture contained 0.1 *M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, 5 m*M* ATP, 100 m*M* glucose,

0.4 m*M* NADP, and 0.3 U glucose 6-phosphate dehydrogenase. 6-Phosphofructokinase activity was analyzed according to Karadsheh *et al.* (17). The assay mixture contained 50 m*M* Tris-HCl (pH 8.2), 1 m*M* fructose 6-phosphate, 1 m*M* ATP, 0.16 m*M* NADH, 1 m*M* EDTA, 2.5 m*M* DTT, 2 m*M* $MgCl_2$, 5 m*M* ammonium sulfate, 0.4 U aldolase, 2.4 U triosephosphate isomerase, and 0.4 U glycerophosphate dehydrogenase. Pyruvate kinase was examined according to the method of described by Harada *et al.* (18). The assay mixture contained 50 m*M* Tris-HCl (pH 7.5), 100 m*M* KCl, 5 m*M* $MgSO_4$, 2 m*M* phosphoenolpyruvate, 2 m*M* ADP, 0.5 m*M* fructose 1,6-bisphosphate, 0.18 m*M* NADH, and 8 U lactate dehydrogenase. Phosphoenolpyruvate carboxykinase was assayed according to the method of Petrescu *et al.* (19). The assay mixture contained 50 m*M* Tris-HCl (pH 7.4), 20 m*M* $NaHCO_3$ (CO_2 saturated), 0.5 m*M* phosphoenolpyruvate, 1 m*M* $MnCl_2$, 0.1 m*M* NADH, 2 U malate dehydrogenase and 0.2 m*M* dGTP. Fructose 1,6-bisphosphatase was analyzed using the method of Tejwani *et al.* (20). The assay mixture contained 100 m*M* Tris-HCl (pH 7.5), 2 m*M* $MgCl_2$, 40 m*M* ammonium sulfate, 0.15 m*M* fructose 1,6-bisphosphate, 0.3 m*M* NADP, 0.1 m*M* EDTA and 0.7 U glucose 6-phosphate dehydrogenase. Glucose 6-phosphatase (EC 3.1.3.9) was assayed as described by Sculze *et al.* (21) and defined as the inorganic phosphate from glucose 6-phosphate of 1 μmol liberated/min at 30°C.

Total glycogen synthase activity (EC 2.4.1.11) was measured by the method of Danforth (22). The enzyme activity was assayed at pH 7.4 and 30°C in a reaction mixture containing 60 m*M* Tris-HCl (pH 7.4), 1.2 m*M* EDTA, 3 m*M* mercaptoethanol, 1.2 m*M* NaF, 7.5 m*M* uridine 5′-diphosphate (UDP)-glucose and 1.2% glycogen. The assay was carried out in the presence 12 m*M* glucose 6-phosphate to measure total glycogen synthase activity. The reaction was terminated by heating for 2 mins in a boiling water bath. The denatured protein was removed by centrifugation and the supernatant solution was assayed enzymatically for UDP. UDP was measured by allowing UDP to react with phosphoenolpyruvate in the presence of pyruvate kinase.

Alanine aminotransferase (EC 2.6.1.2) and aspartate aminotransferase (EC 2.6.1.1) were assayed according to the methods described by Karmen *et al.* (23) and Wroblewski *et al.* (24), respectively. The assay mixture for alanine aminotransferase contained 80 m*M* sodium phosphate buffer (pH 7.4), 80 m*M* DL-alanine, 18 m*M* α-ketoglutarate, 0.12 m*M* NADH and 1400 U/ml lactate dehydrogenase. The assay mixture for aspartate aminotransferase contained 80 m*M* sodium phosphate buffer (pH 7.4), 125 m*M* L-aspartate, 12 m*M* α-ketoglutarate, 0.12 m*M* NADH, 160 U/ml malate dehydrogenase and 300 U/ml lactate dehydrogenase.

The total protein concentration of the liver homogenate supernatant was measured using bicinchoninic acid with bovine serum albumin as the standard (25).

Amino Acids Analyses. Free amino acids were determined by high-performance liquid chromatography (HPLC), using a postcolumn fluorometric detection system and the *o*-phthalaldehyde/N-acetyl-L-cysteine reagent (26). The amino acid composition of the three dietary proteins was determined after hydrolysis in 6 *N* HCl. Serum proteins were removed with 5% trichloroacetate, and after neutralization, the samples of serum free amino acids were subjected to HPLC. The amino acid composition of the experimental diets is shown in Table 2.

Statistics. Data of the exercise-trained and sedentary rats fed casein were compared using Student's *t* test, while data of the three exercise-trained groups were analyzed by one-way ANOVA with post hoc analyses being carried out using Duncan's multiple range test. Differences between groups were considered to be statistically significant at $P < 0.05$.

Results

Food Intake, Body Weight Gain, and Liver Weight. Initial body weight, food intake, and body weight gain are shown in Table 3. Although food intake in the exercise-trained rats decreased significantly, this had no effect on body weight gain or liver weight compared with sedentary rats. Food intake, body weight gain, and liver weight were similar in the three exercise-trained groups.

Serum Glucose, Insulin, and Glucagon. Serum glucose, insulin and glucagon levels were similar in the exercise-trained and sedentary rats. In exercise-trained rats, these parameters did not differ among three groups (Table 4).

Liver Glycogen Content. The level of liver glycogen was significantly higher in the exercise-trained rats than in sedentary rats. In the exercise-trained rats, significantly higher levels of hepatic glycogen content were observed in the group fed whey protein compared to the groups fed either casein or soy protein (Fig. 1).

Table 2. Amino Acid Composition of the Three Dietary Proteins (mg/100 mg Protein)

	Casein	Whey	Soy
Ala	2.79	4.91	4.28
Arg	3.53	2.21	7.74
Asp+Asn	6.51	10.73	11.46
Cys	0.37	1.91	1.31
Glu+Gln	20.63	17.55	18.54
Gly	1.95	1.91	4.12
His	2.97	2.11	2.53
Ile	4.74	5.12	4.54
Leu	8.74	12.04	8.09
Lys	7.16	9.23	6.26
Met	2.60	2.51	1.32
Phe	4.55	3.51	5.41
Pro	11.90	6.22	5.10
Ser	5.58	3.71	5.42
Thr	3.81	4.91	3.93
Trp	1.12	1.91	1.38
Tyr	5.11	3.81	3.86
Val	5.95	5.72	4.70

Table 3. Initial Body Weight, Food Intake, Body Weight Gain, and Liver Weight in the Four Groups of Rats[a]

	Sedentary	Exercise		
Group	Casein	Casein	Whey protein	Soy protein
Initial body weight (g)	130 ± 3	129 ± 3	130 ± 3	129 ± 5
Food intake (g/4 weeks)	625 ± 9***	571 ± 7	550 ± 11	576 ± 16
Body weight gain (g/4 weeks)	194 ± 8	180 ± 5	168 ± 5	166 ± 5
Liver weight (g/100 g body weight)	3.03 ± 0.06	3.14 ± 0.11	3.17 ± 0.06	2.95 ± 0.06

[a] Data are expressed as means ± SE. Asterisks indicate significant differences between exercise-trained rats fed casein and sedentary rats fed casein (***$P < 0.001$).

Liver Enzyme Activities. Exercise training did not cause any change in liver enzyme activity. In the exercise-trained groups, glucokinase activity was significantly higher in rats fed whey protein diet than in rats given the soy protein diet, whereas glucose 6-phosphatase activity in the whey protein group was significantly decreased compared to the casein and soy groups. Although 6-phosphofructokinase activity was lower in the whey protein group compared to the soy protein group, fructose 1,6-bisphosphatase activity was significantly higher in rats fed whey protein compared to the other two diets. Pyruvate kinase activity in rats fed the casein-containing diet was significantly higher than in rats fed either whey or soy protein, while phosphoenolpyruvate carboxylase activity was similar in the three groups (Table 5).

In the exercise-trained rats, alanine aminotransferase activity was significantly higher in the whey protein group than in the other two groups, whereas aspartate aminotransferase activity did not differ between the groups (Fig. 2).

Serum Free Amino Acids. A significant increase in serum free asparagine, glutamine, histidine, isoleucine, leucine, lysine, proline, threonine, tyrosine, and valine was observed in the exercise-trained rats compared with the sedentary rats. Total free amino acids (TAAs), total essential free amino acids (EAAs), and branched chain amino acids (BCAAs) were also increased by exercise training. We also observed that the type of dietary protein also affected the level of several serum free amino acids. Exercise-trained rats fed whey protein had significantly higher serum levels of alanine, arginine, and glycine than animals fed casein, while rats fed casein had significantly higher asparagine, glutamine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, tyrosine, TAAs, EAAs, and BCAAs levels than rats fed soy protein (Table 6).

Discussion

The depletion of glycogen stores has been associated with fatigue during both sprint and endurance exercise (9–11, 27). Therefore, it is of considerable importance to athletes that they have adequate stores of glycogen in tissues. However, it remains unclear whether the type of dietary protein affects glycogen content or glycoregulatory enzyme activities in tissues. This study showed for the first time that the type of dietary protein affects liver glycogen content. This effect was especially apparent in the exercise-trained rats fed a whey protein diet, with these animals storing considerably more glycogen in the liver than similar rats fed either a casein or soy protein diet.

In the liver, three key enzyme sites regulate the interaction between the glycolytic and gluconeogenic pathways. These include glucokinase/glucose 6-phosphatase, 6-phosphofructokinase/fructose 1,6-bisphosphatase, and pyruvate kinase/phosphoenolpyruvate carboxykinase. The three reactions involved in glycolysis proceed with a large negative free energy change and are therefore bypassed during gluconeogenesis by the use of the alternative enzymes. In this study, we observed that glucokinase activity was significantly higher in animals fed whey protein than animals fed soy protein, whereas the whey protein diet led to significantly lower hepatic glucose 6-phosphatase activity than the casein and soy diets. Whey protein was also associated with significantly lower 6-phosphofructokinase activity than soy protein, and significantly higher fructose 1,6-bisphosphatase activity than casein or soy protein. In addition, we found that pyruvate kinase activity in rats fed the casein-containing diet was significantly higher than in rats fed either whey or soy protein, while phosphoenolpyruvate carboxylase activity was similar with all three diets. Recently, de la Iglesia *et al.* (28) showed that glucokinase

Table 4. Serum Glucose, Insulin, and Glucagon in the Four Groups of Rats[a]

	Sedentary	Exercise		
Group	Casein	Casein	Whey protein	Soy protein
Glucose (g/l)	1.07 ± 0.07	1.12 ± 0.09	1.30 ± 0.12	1.10 ± 0.08
Insulin (ng/l)	894 ± 370	640 ± 198	940 ± 188	522 ± 80
Glucagon (ng/l)	1520 ± 62	1374 ± 120	1217 ± 100	1266 ± 145

[a] Data are expressed as means ± SE.

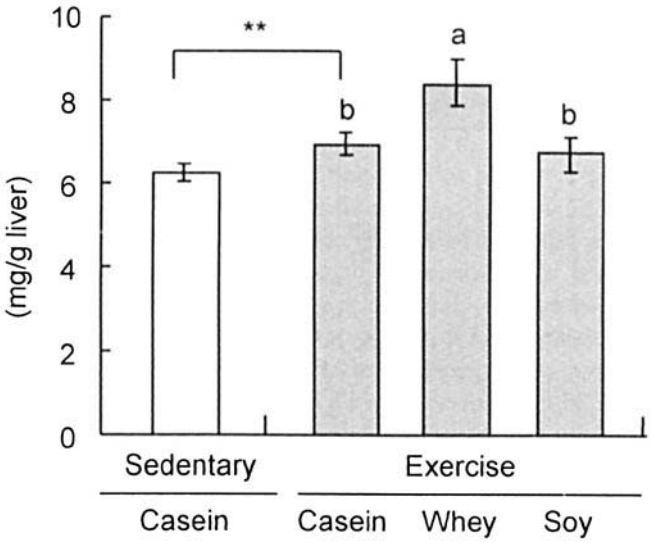

Figure 1. Liver glycogen content. Data are expressed as means ± SE. Asterisks indicate significant differences between exercise-trained rats fed casein and sedentary rats fed casein (**$P < 0.01$). In the three exercise-trained groups, means without a common letter are significantly different, $P < 0.05$.

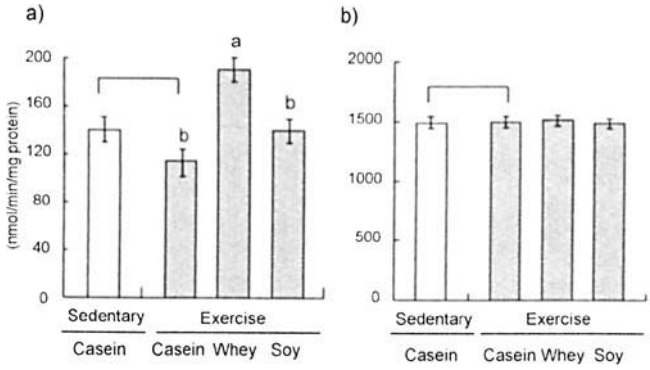

Figure 2. Liver aminotransferase activities, (a) alanine aminotransferase, (b) aspartate aminotransferase. Data are expressed as means ± SE. In the three exercise-trained groups, means without a common letter are significantly different, $P < 0.05$.

has a number of unique regulatory properties that exert considerable control over hepatic glucose uptake and glycogen synthesis. In our study however, liver glycogen levels were significantly higher in the whey group than in either the casein and soy groups, while glucokinase and 6-phosphofructokinase activity, both key enzymes in the regulation of glycolysis, were similar in the casein and whey protein groups. During starvation or caloric restriction, pathways associated with glucose synthesis (i.e., gluconeogenesis and amino acid catabolism) are stimulated, while those associated with glucose catabolism (i.e., glycolysis and the Krebs cycle) are inhibited (29–31). It has been shown in both rodents and humans that this adaptation occurs in response to decreased serum levels of glucose and insulin (32, 33), with regulation of hepatic glycolytic and gluconeogenic enzymes being mediated primarily by insulin. Therefore, after a 12-hr fast, the beneficial effects of glucokinase on control of hepatic glucose uptake and glycogen synthesis may be diminished. However, the activity of key gluconeogenic enzymes, such as fructose 1,6-bisphosphatase and glucose 6-phosphatase, may play a more important role in hepatic glycogen storage. This possibility is supported by our data that showed clearly that glucose 6-phosphatase activity was significantly lower and fructose 1,6-bisphosphatase activity significantly higher in the liver of rats fed whey protein compared with rats given casein or soy protein. Therefore, in the fasted state, the activity of key enzymes involved in the regulation of the gluconeogenic pathway rather than the glycolytic pathway appears to determine the level of hepatic glycogen. However, the mechanism by which gluconeogenic enzyme activity determines glyconeogenesis has yet to be established.

In our study, we found that the serum levels of glucose, insulin, and glucagon did not differ in the three exercise-trained groups. Despite these similarities, dietary whey protein modulated the activity of various hepatic glycolytic and gluconeogenic enzymes in these animals. There is evidence that certain amino acids stimulate glycolytic and gluconeogenic enzyme activity, with Aoyama *et al.* (34, 35) showing that dietary excess of histidine led to increased hepatic glycogen content as a result of activation of fructose 1,6-bisphosphatase, and suppression of glucose 6-phosphatase, 6-phosphofructokinase, and pyruvate kinase activities. Studies in isolated hepatocytes also demonstrated that

Table 5. Liver Glycoregulatory Enzyme Activities in the Four Groups of Rats (nmol/min/mg Protein)[a]

	Sedentary	Exercise		
Group	Casein	Casein	Whey protein	Soy protein
Glucokinase	13.19 ± 1.23	13.34 ± 0.76^{AB}	14.03 ± 0.75^{A}	11.61 ± 0.71^{B}
6-Phosphofructokinase	14.09 ± 0.70	12.68 ± 0.70^{AB}	11.02 ± 0.59^{B}	13.67 ± 0.83^{A}
Pyruvate kinase	1090 ± 57	1004 ± 62^{A}	842 ± 40^{B}	791 ± 61^{B}
Glucose 6-phosphatase	54.9 ± 4.7	49.9 ± 2.7^{A}	37.8 ± 3.0^{B}	47.6 ± 2.5^{A}
Fructose 1,6-bisphosphatase	111.5 ± 3.0	111.9 ± 2.5^{B}	123.7 ± 3.2^{A}	109.9 ± 4.2^{B}
Phosphoenolpyruvate carboxykinase	18.78 ± 1.36	19.06 ± 0.68	18.65 ± 0.94	16.85 ± 1.66
Total glycogen synthase	1.13 ± 0.18	1.21 ± 0.20	1.29 ± 0.16	1.19 ± 0.22

[a] Data are expressed as means ± SE. In the three exercise-trained groups, means without a common superscript letter are significantly different, $P < 0.05$.

Table 6. Serum Amino Acids (μM) in the Four Groups of Rats[a]

	Sedentary	Exercise		
Group	Casein	Casein	Whey protein	Soy protein
Alanine	489 ± 30	540 ± 27^{B}	645 ± 50^{A}	465 ± 27^{B}
Arginine	233 ± 12	224 ± 10^{B}	262 ± 7^{A}	243 ± 16^{AB}
Asparagine	82.8 ± 3.1*	91.9 ± 3.2^{A}	92.1 ± 2.4^{A}	75.7 ± 2.6^{B}
Aspartate	ND	ND	ND	ND
Cystine	ND	ND	ND	ND
Glutamine	841 ± 33**	959 ± 30^{A}	980 ± 37^{A}	815 ± 25^{B}
Glutamate	93.0 ± 8.2	112 ± 13	102 ± 13	93.6 ± 10.0
Glycine	335 ± 14	368 ± 22^{B}	433 ± 11^{A}	404 ± 25^{AB}
Histidine	59.5 ± 1.7**	68.9 ± 2.8^{A}	68.1 ± 0.6^{A}	58.1 ± 2.0^{B}
Isoleucine	131 ± 5*	151 ± 6^{A}	155 ± 5^{A}	125 ± 5^{B}
Leucine	191 ± 8*	219 ± 9^{A}	233 ± 6^{A}	179 ± 10^{B}
Lysine	533 ± 32*	668 ± 48^{A}	641 ± 21^{AB}	557 ± 22^{B}
Methionine	73.0 ± 2.2	75.9 ± 2.5^{AB}	81.6 ± 4.3^{A}	69.1 ± 1.5^{B}
Phenylalanine	80.3 ± 2.7	88.6 ± 4.0^{A}	90.5 ± 3.6^{A}	74.0 ± 3.1^{B}
Proline	182 ± 5*	199 ± 4^{A}	197 ± 6^{A}	173 ± 6^{B}
Serine	335 ± 15	337 ± 8	369 ± 19	369 ± 23
Threonine	346 ± 13**	433 ± 22^{A}	327 ± 19^{B}	381 ± 32^{AB}
Tryptophane	81.1 ± 7.2	87.4 ± 4.4	96.7 ± 5.6	85.4 ± 7.1
Tyrosine	112 ± 4***	142 ± 5^{A}	116 ± 8^{B}	119 ± 4^{B}
Valine	215 ± 10**	245 ± 8^{A}	257 ± 5^{A}	229 ± 6^{B}
TAAs	4411 ± 108**	5009 ± 136^{A}	5146 ± 112^{A}	4486 ± 119^{B}
EAAs	1709 ± 58**	2036 ± 81^{A}	1950 ± 17^{A}	1731 ± 49^{B}
BCAAs	536 ± 23*	615 ± 21^{A}	646 ± 15^{A}	573 ± 14^{B}

[a] Data are expressed as means ± SE. ND, not detected. Asterisks indicate significant differences between exercise-trained rats fed casein and sedentary rats fed casein (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). In the three exercise-trained groups, means without a common superscript letter are significantly different, $P < 0.05$.

several amino acids including glutamine, proline, alanine, and histidine stimulated glycogen synthesis from both glucose and gluconeogenic precursors (36). In addition, it is known that whey protein has a high concentration of branched chain amino acids (BCAAs), and there is a report indicating that BCAAs supplements reduced glycogen consumption during acute exercise as a consequence of decreased activity of the pyruvate dehydrogenase complex (37). We consider it is therefore likely that the beneficial effect of whey protein on hepatic glycogen storage is determined by the amino acid composition of the protein, although the manner by which this effect is exerted is not fully understood.

Although several studies have shown that exercise training increases hepatic glycogen content (38–40), the reported effects of training on liver glycogen levels are variable and remain controversial. It is possible that trained individuals may have increased endurance by starting the exercise period with higher liver glycogen stores or by maintaining high liver glycogen levels throughout the period of exercise (41). In our study we showed that liver glycogen increased with exercise training, while liver total glycogen synthase activity was not affected by treadmill-exercise training. These findings are similar to those reported by Campell *et al.* (38) but differ from those of James and Kraegen (42) who demonstrated that rats trained by a treadmill running protocol involving 120 min/day for 5 days/week at grade 15 did not have increased resting liver glycogen levels or total glycogen synthase activity over and above that measured in sedentary controls. In contrast, studies in rats using a training protocol involving swimming 6 hours/day, 5 days/week for 14 weeks reported increased resting liver glycogen concentration of up to 75% in trained rats compared with sedentary controls (43). It is likely that this variability in findings between the studies may reflect the different types and intensity of exercise used to train the animals. Notwithstanding this possibility, the mechanism responsible for the increase in hepatic glycogen associated with exercise training is yet to be defined conclusively.

It is well established that the level of amino acids in the serum of animals may be modulated by exercise training or by different types of dietary protein. Dohm *et al.* (44) measured the amino acid composition in rat plasma immediately after swimming exercise and observed a decrease in the concentration of alanine, asparagine, aspartate, arginine, glutamate, glutamine, glycine, histidine, methionine, proline, serine, and threonine, and an increase in the concentration of valine and leucine. Although these earlier studies measured the level of serum amino acids immediately before, during, and after exercise (44, 45), in our study we investigated whether long-term exercise training affected serum amino acid concentration in the resting state. We observed significant increases in the serum levels of free asparagine, glutamine, histidine, isoleucine, leucine, lysine, proline, threonine, tyrosine, and valine in

exercise-trained rats compared with sedentary rats. Exercise training was also shown to increase the level of TAAs, EAAs, and BCAAs in our rats. Exercise results in the loss of liver protein and increased muscle protein degradation, and the generalized increase we observed in the level of serum essential amino acids is confirmation that protein degradation is increased by a bout of exercise (4). As BCAAs comprise a significantly large amount of essential amino acids in muscle protein, the marked increase in the level of serum BCAAs that occurred in our study was probably a result of degradation of muscle protein. It is also likely that differences in BCAAs content in the three dietary proteins may have also contributed to the increase in serum BCAAs, as whey and casein contain more BCAAs than soy protein, a difference reflected by the higher levels of BCAAs we observed in the casein and whey groups.

This study showed that compared to casein, whey protein caused significant increases in the level of various amino acids, especially alanine and also enhanced hepatic alanine aminotransferase activity. Transamination is the first step in the catabolism of most amino acids and allows the carbon skeleton of the amino acids to be converted to oxaloacetate and subsequently to pyruvate that is then utilized by the gluconeogenic pathway. When glycogen stores are depleted in muscle during exertion and in liver during fasting, catabolism of muscle proteins to form amino acids constitutes a major source of carbon for the maintenance of blood glucose levels. Felig and Wahren (5) demonstrated that alanine was released from muscles in greater amounts during exercise, leading to increased plasma concentrations and greater uptake of alanine by the liver and therefore increased gluconeogenesis. This is termed the glucose–alanine cycle. In our study, it is possible that BCAAs may have been converted to alanine in skeletal muscle and then transported to the liver for conversion to pyruvate, thereby reducing the need to metabolize liver glycogen and produce additional glucose. The findings of our study are in accordance with this sequence of reactions, as whey protein, in addition to increasing the level of alanine in serum, also increased the activity of hepatic alanine aminotransferase. This increase in alanine aminotransferase indicated enhancement glycogenesis from alanine.

In summary, we demonstrated in exercise-trained rats that a whey protein diet, compared with casein and soy protein diets, had beneficial effects on liver glycogen storage as a result of regulation of the activity of glycolytic and gluconeogenic enzymes and aminotransferase. In addition, our results showed that exercise-trained rats had significantly decreased food intake compared to sedentary rats. Taken together, these results indicate that whey protein may have a beneficial role in maintaining stores of glycogen during conditions of energy deficit. We consider that daily intake of whey protein has potential to not only stimulate and maintain muscle growth and strength but also maintain a high level of glycogen in the liver.

1. Lemaigre FP, Rousseau GG. Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult liver. Biochem J 303:1–14, 1994.
2. Newsholme EA, Start C. Regulation in Metabolism. New York: Wiley, pp146–194, 329–337, 1981.
3. Nordlie RC, Foster JD, Lange AJ. Regulation of glucose production by the liver. Annu Rev Nutr 19:379–406, 1999.
4. Kasperek GJ, Dohm GL, Barakat HA, Strausbauch PH, Barnes DW, Snider RD. The role of lysosomes in exercise-induced hepatic protein loss. Biochem J 202:281–288, 1982.
5. Felig P, Wahren J. Protein turnover and amino acid metabolism in the regulation of gluconeogenesis. Fed Proc 33:1092–1097, 1974.
6. Lemon PW. Protein and amino acid needs of the strength athlete. Int J Sport Nutr 1:127–145, 1991.
7. Yoshizawa F. Regulation of protein synthesis by branched-chain amino acids in vivo. Biochem Biophys Res Commun 313:417–422, 2004.
8. Nishitani S, Ijichi C, Takehana K, Fujitani S, Sonaka I. Pharmacological activities of branched-chain amino acids: specificity of tissue and signal transduction. Biochem Biophys Res Commun 313:387–389, 2004.
9. Terjung RL, Baldwin KM, Winder WW, Holloszy JO. Glycogen repletion in different types of muscle and in liver after exhausting exercise. Am J Physiol 226:1387–1391, 1974.
10. Hermansen L, Hultman E, Saltin B. Muscle glycogen during prolonged severe exercise. Acta Physiol Scand 71:129–139, 1967.
11. Karlsson J, Saltin B. Diet, muscle glycogen, and endurance performance. J Appl Physiol 31:203–206, 1971.
12. Krahenbuhl L, Lang C, Ludes S, Seiler C, Schafer M, Zimmermann A, Krahenbuhl S. Reduced hepatic glycogen stores in patients with liver cirrhosis. Liver Int 23:101–109, 2003.
13. Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951, 1993.
14. Miwa I, Okudo J, Maeda K, Okuda G. Mutarotase effect on colorimetric determination of blood glucose with D-glucose oxidase. Clin Chim Acta 37:538–540, 1972.
15. Lo S, Russell JC, Taylor AW. Determination of glycogen in small tissue samples. J Appl Physiol 28:234–236, 1970.
16. Grossman S, Dorn C, Potter V. The preparation and characterization of pure rat liver glucokinase. J Biol Chem. 249:3055–3060, 1974.
17. Karadsheh NS, Uyeda K, Oliver RM. Studies on structure of human erythrocyte phosphofructokinase. J Biol Chem 252:3515–3524, 1977.
18. Harada K, Saheki S, Wada K, Tanaka T. Purification of four pyruvate kinase isozymes of rats by affinity elution chromatography. Biochim Biophys Acta 524:327–339, 1978.
19. Petrescu I, Bojan O, Saied M, Barzu O, Schmidt F, Kuhnle HF. Determination of phosphoenolpyruvate carboxykinase activity with deoxyguanosine 5′-diphosphate as nucleotide substrate. Anal Biochem 96:279–281, 1979.
20. Tejwani GA, Pedrosa FO, Pontremoli S, Horecker BL. The purification of properties of rat liver fructose 1,6-bisphosphatase. Arch Biochem Biophys 177:253–264, 1976.
21. Schulze HU, Kannler R, Junker B. Latency studies on rat liver microsomal glucose-6-phosphatase. Correlation of membrane modification and solubilization by Triton X-114 with the enzymatic activity. Biochim Biophys Acta 814:85–95, 1985.
22. Danforth WH. Glycogen synthetase activity in skeletal muscle. Interconversion of two forms and control of glycogen synthesis. J Biol Chem 240:588–593, 1965.
23. Karmen A, Wroblewski F, LaDue JS. Transaminase activity in human blood. J. Clin. Invest. 94:126–131, 1955.
24. Wroblewski F, LaDue JS. Serum glutamic pyruvic transaminase in cardiac and hepatic disease. Proc Soc Exp Biol Med 91:567–571, 1956.

25. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85, 1985.
26. Fujiwara M, Ishida Y, Nimura N, Toyama A, Kinoshita T. Postcolumn fluorometric detection system for liquid chromatographic analysis of amino and imino acids using o-phthalaldehyde/N-acetyl-L-cysteine reagent. Anal Biochem 166:72–78, 1987.
27. Newsholme EA, Blomstrand E, Ekblom B. Physical and mental fatigue: metabolic mechanisms and importance of plasma amino acids. Br Med Bull 48:477–495, 1992.
28. de la Iglesia N, Mukhtar M, Seoane J, Guinovart JJ, Agius L. The role of the regulatory protein of glucokinase in the glucose sensory mechanism of the hepatocyte. J Biol Chem 275:10597–10603, 2000.
29. Friedman WJ, Dreyfus CF, McEwen BS, Black IB. Developmental regulation of tyrosine hydroxylase in the mediobasal hypothalamus. Brain Res Dev Brain Res 48:177–185, 1989.
30. Hagopian K, Ramsey JJ, Weindruch R. Caloric restriction increases gluconeogenic and transaminase enzyme activities in mouse liver. Exp Gerontol 38:267–278, 2003.
31. Hagopian K, Ramsey JJ, Weindruch R. Influence of age and caloric restriction on liver glycolytic enzyme activities and metabolite concentrations in mice. Exp Gerontol 38:253–266, 2003.
32. Harris SB, Gunion MW, Rosenthal MJ, Walford RL. Serum glucose, glucose tolerance, corticosterone and free fatty acids during aging in energy restricted mice. Mech Ageing Dev 73:209–221, 1994.
33. Walford RL, Harris SB, Gunion MW. The calorically restricted low-fat nutrient-dense diet in Biosphere 2 significantly lowers blood glucose, total leukocyte count, cholesterol, and blood pressure in humans. Proc Natl Acad Sci U S A 89:11533–11537, 1992.
34. Aoyama Y, Tsuda T, Hitomi-Ohmura E, Yoshida A. Effect of dietary excess-histidine on fructose 1,6-bisphosphatase and 6-phosphofructokinase activities, and activation of fructose 1,6-bisphosphatase by basic amino acids in rat liver. Int J Biochem 24:981–985, 1992.
35. Aoyama Y, Tsuda T, Hitomi-Ohmura E, Yoshida A. Activities of some regulatory enzymes of carbohydrate metabolism in the liver of rats fed a histidine-excess diet. Comp Biochem Physiol Comp Physiol 104:381–388, 1993.
36. Baquet A, Hue L, Meijer AJ, van Woerkom GM, Plomp PJ. Swelling of rat hepatocytes stimulates glycogen synthesis. J Biol Chem 265:955–959, 1990.
37. Shimomura Y, Murakami T, Nakai N, Nagasaki M, Obayashi M, Li Z, Xu M, Sato Y, Kato T, Shimomura N, Fujitsuka N, Tanaka K, Sato M. Suppression of glycogen consumption during acute exercise by dietary branched-chain amino acids in rats. J Nutr Sci Vitaminol (Tokyo) 46:71–77, 2000.
38. Campbell WW, Polansky MM, Bryden NA, Soares JH, Jr., Anderson RA. Exercise training and dietary chromium effects on glycogen, glycogen synthase, phosphorylase and total protein in rats. J Nutr 119:653–660, 1989.
39. Garrido G, Guzman M, Odriozola JM. Effect of different types of high carbohydrate diets on glycogen metabolism in liver and skeletal muscle of endurance-trained rats. Eur J Appl Physiol Occup Physiol 74:91–99, 1996.
40. Murakami T, Shimomura Y, Fujitsuka N, Sokabe M, Okamura K, Sakamoto S. Enlargement glycogen store in rat liver and muscle by fructose-diet intake and exercise training. J Appl Physiol 82:772–775, 1997.
41. Fitts RH, Booth FW, Winder WW, Holloszy JO. Skeletal muscle respiratory capacity, endurance, and glycogen utilization. Am J Physiol 228:1029–1033, 1975.
42. James DE, Kraegen EW. The effect of exercise training on glycogen, glycogen synthase and phosphorylase in muscle and liver. Eur J Appl Physiol Occup Physiol 52:276–281, 1984.
43. Baldwin KM, Fitts RH, Booth FW, Winder WW, Holloszy JO. Depletion of muscle and liver glycogen during exercise. Protective effect of training. Pflugers Arch 354:203–212, 1975.
44. Dohm GL, Beecher GR, Warren RQ, Williams RT. Influence of exercise on free amino acid concentrations in rat tissues. J Appl Physiol 50:41–44, 1981.
45. Henriksson J. Effect of exercise on amino acid concentrations in skeletal muscle and plasma. J Exp Biol 160:149–165, 1991.