



Basic nutritional investigation

Dietary whey hydrolysate with exercise alters the plasma protein profile: A comprehensive protein analysis

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ABSTRACT

Objective: It has been shown that dietary whey protein accelerates glucose uptake by altering glycoregulatory enzyme activity in skeletal muscle. In the present study, we investigated the effect of dietary whey protein on endurance and glycogen resynthesis and attempted to identify plasma proteins that reflected the physical condition by a comprehensive proteomics approach.

Methods: Male c57BL/6 mice were divided into four groups: sedentary, sedentary with whey protein hydrolysate, exercise, and exercise with whey protein hydrolysate. The mice in the exercise groups performed treadmill running exercise five times per week for 4 wk. Protein profiling of plasma sample obtained from individuals was performed, as were measurements of endurance performance and the glycogen content of gastrocnemius muscle.

Results: After the training period, the endurance of mice fed the whey diet was improved compared with that of mice fed the control diet. Muscle glycogen content was significantly increased after 4 wk of exercise, and intake of whey protein led to a further increase in glycogen. Apolipoproteins A-II and C-I and β_2 -glycoprotein-1 were found to be altered by training combined with the intake of whey protein, without significant changes induced by exercise or whey protein alone.

Conclusion: Results of the present study suggest that these three proteins may be potential biomarkers of improved endurance and glycogen resynthesis and part of the mechanism that mediates the benefits of whey protein.

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Introduction

Whey protein accounts for about 20% of bovine milk protein and mainly consists of α -lactoglobulin, β -lactalbumin, albumin, and immunoglobulins [1]. It contains high concentrations of branched-chain amino acids, which promote protein synthesis by inhibition of protein degradation and being constituents of

muscle protein [2]. In addition, whey protein is well digested and easily absorbed, resulting in a rapid increase in the blood level of amino acids [3,4]. Therefore, supplementation with whey protein has been used by athletes to promote and maintain muscle growth and strength. Dietary intake of whey protein also affects glucose metabolism in the skeletal muscles. It has been shown that ingestion of carbohydrates with whey protein accelerates glucose uptake by increasing glycoregulatory enzyme activity in muscle, and the effect is greater compared with that of dietary casein or soy [5–7].

During endurance exercise, glycogen (an energy substrate used for muscle contraction) is gradually depleted, making it difficult to continue exercise. An effective way to improve endurance is to increase the glycogen stores in skeletal muscle

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and the liver before the start of exercise. It is also important for athletes to replenish their glycogen stores during postexercise training to provide sufficient energy for the next training session or for competition. Because muscle glycogen resynthesis is relatively slow and can take around 24 h for complete replenishment of muscle glycogen, several studies have been conducted to determine ways to enhance the rate of muscle glycogen resynthesis [8,9]. Although eating a high-carbohydrate diet after exercise is effective for rapid replenishment of glycogen stores, intake of whey protein and carbohydrate can be more effective for replenishment of muscle glycogen after exercise compared with carbohydrate supplementation alone [10]. Therefore, intake of whey protein may lead to greater improvement of endurance with exercise training.

The proteins circulating in the blood reflect various physiologic and pathologic conditions. Recently, proteomics technique has made great progress and identification of new proteins has been carried out in various conditions. It has been reported that several blood proteins are useful as biomarkers for predicting various physiologic and pathologic conditions, such as cancer, diabetes, and cardiovascular disease [11–16]. However, a proteomics approach to sports science has not been attempted, although it is generally known that exercise alters various blood components, including glucose, lipids, and hormones. In the present study, we employed a comprehensive proteomics approach to identify blood proteins associated with the improvement of endurance and glycogen resynthesis by muscle. If changes of proteins in the blood due to exercise were promoted by the intake of whey protein, such proteins could be useful as biomarkers of endurance and glycogen resynthesis capacity by muscle. In addition, such changes of blood proteins may help to explain the improvement of glucose metabolism in skeletal muscle by dietary intake of whey protein. In this study, we found three proteins that were altered by training with dietary intake of whey protein.

Materials and methods

Animals and experimental design

The present study complied with the principles and guidelines of the Japanese Council on Animal Care and it was approved by the committee for animal research of Kyoto Prefectural University of Medicine (permission no. M19-42). C57BL/6 mice (8 wk old) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and were acclimatized for 1 wk in an air-conditioned (22 ± 2°C) room with a 12-h light/dark cycle (lights on from 08:00 to 20:00 h). The mice were divided into four groups of eight animals each, consisting of a sedentary plus control diet group (SC), a sedentary plus whey diet group (SW), an exercise training plus control diet group (TC), and an exercise training plus whey diet group (TW). The design of the experimental diets followed the AIN-93 protocol, and the composition of the diets is presented in Table 1. Whey diet was prepared by exchanging casein in the control diet to whey protein hydrolyzed with *Aspergillus oryzae* and *Bacillus subtilis* proteases at TATUA Co., Ltd. (Morrinsville, New Zealand). Casein (87.7 g of crude protein/100 g) and whey protein (79.3 g of crude protein/100 g) were counted as 200 g of protein per 1 kg to the diet. The difference in the protein content between the two diets was compensated for by the addition of cornstarch. The training groups

were allowed to run on a motorized treadmill five times per week for 4 wk. During the initial week, the level of exercise was gradually increased from running for 15 min with the treadmill set at 15 m/min to running for 30 min with the treadmill set at 22 m/min, which was above the estimated blood lactate threshold [17,18]. Then the speed was kept at 22 m/min for the following 3 wk. At the end of the 3 wk of training, endurance performance testing was performed. In the test, the running speed was gradually increased to 30 m/min over the initial 5 min and then maintained at that level until exhaustion, while the running time to exhaustion was measured. Exhaustion was defined as the inability of a mouse to right itself after being placed on its side. No mouse ceased exercise because of injury. After the endurance test, the running training was continued for 1 more week. The mice were euthanized at 24 h after the last training session, and the gastrocnemius muscle was rapidly removed for glycogen measurement, and blood was collected for proteomics analysis.

Glycogen measurement

Muscle 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 [19].

Protein profiling using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI TOF MS)

Plasma samples were thawed on ice and centrifuged at 20 000 × g for 10 min. All samples were divided into two 20-μL aliquots, denatured by adding 30 μL of U9 buffer (50 mM Tris-HCl [pH 9.0], 9 M urea, 2% 3-(3-cholamidopropyl)dithylammonio-1-propanesulphonate [CHAPS]), and agitated for 30 min at 4°C. Denatured samples were applied to the wells of a Silent Screen Plate (Nalge Nunc International K.K., Tokyo, Japan) that contained 90 μL of Q Sepharose anion exchange resin (GE Healthcare Bio-Sciences, Buckinghamshire, UK), 50 μL of U1 buffer (U9 buffer diluted nine-fold with 50 mM Tris-HCl [pH 9.0]) was added to each well, and incubation was performed at 4°C for 30 min. After incubation, the flow-through fraction was collected. Subsequent fractions were collected by applying 100 μL of 50 mM Tris-HCl buffer (pH 9.0) and two different buffers with lower pH values: 100 mM sodium acetate buffer (pH 5.0) and 50 mM sodium citrate buffer (pH 3.0). Remaining proteins were eluted by washing the resin with an organic solution composed of 33.3% isopropanol, 16.7% acetonitrile (ACN), and 0.1% trifluoroacetic acid. In summary, four fractions (i.e., flow-through + pH 9.0, pH 5.0, pH 3.0, and organic) were obtained and are referred to as Fr1 to Fr4.

All fractions were subjected to two different types of ProteinChip arrays (Bio-Rad, Richmond, CA, USA), CM10 (weak cation exchange array), and Q10 (strong anion exchange array). The binding buffers for CM10 and Q10 arrays were 50 mM sodium citrate (pH 3.0) and 50 mM Tris-HCl (pH 9.0), respectively. Aliquots (10 μL) of the fractionated eluates were diluted 10-fold with each binding buffer and applied to the ProteinChip arrays. α-Cyano-4-hydroxycinnamic acid and sinapine acid were used as energy absorbing molecules (EAMs). One microliter of 50% saturated EAM solution (in 50% ACN and 0.5% trifluoroacetic acid) was applied to the preprepared arrays. Procedures from sample denaturation to EAM application were performed using a Biomek 2000 automated system (Beckman Coulter Instruments, Inc., Fullerton, CA, USA).

All arrays were analyzed using a Protein Biological System IIc ProteinChip reader (Bio-Rad). The high mass was set to 100 kDa and the focus mass was set to 6.5 kDa. In addition, when sinapine acid was used as an EAM, arrays were further analyzed using the following parameters: high mass set to 200 kDa and focus mass set to 20 kDa. Mass spectrometric profiles were generated by averaging 130 laser shots at optimized analyzing conditions, on the basis of maximum protein peak yield.

Discovery of plasma protein changed by training plus whey protein hydrolysate

At first, with the purpose of discovering protein biomarkers associated with exercise performance, data were analyzed in four steps: step 1, normalized intensities of protein/peptide peaks obtained by SELDI-TOF MS were comprehensively compared between the SC and TC groups; step 2, artifacts (noise, multiply charged peaks, and matrix adduct peaks) were removed from the peaks selected in step 1 and redundant peaks derived from the same protein were grouped; step 3, normalized intensities of protein/peptide peaks obtained by SELDI-TOF MS were comprehensively compared between the TW and TC groups in relation to candidates identified in step 1; step 4, peaks with an opposite direction than those elicited by training were removed from the peaks selected in step 3 (Fig. 1). This analysis did not result in the identification of protein biomarkers influenced by training and whey. Therefore, we searched for proteins that changed in response to exercise training in the TW group, but did not change in the other three groups. As above, data were analyzed in four steps: step 1–2, normalized intensities of protein/peptide peaks obtained by SELDI-TOF MS were comprehensively compared between the TC and TW groups; step 2–2, artifacts (noise, multiply charged peaks, and matrix adduct peaks) were removed from the

Table 1
Composition of the control and whey diets

	Control diet (g/kg)	Whey diet (g/kg)
Casein	228	–
Whey protein	–	252
Vitamin mixture	10	10
Choline bitartrate	2.5	2.5
Mineral mixture	35	35
Corn oil	70	70
Corn starch	654.5	630.5

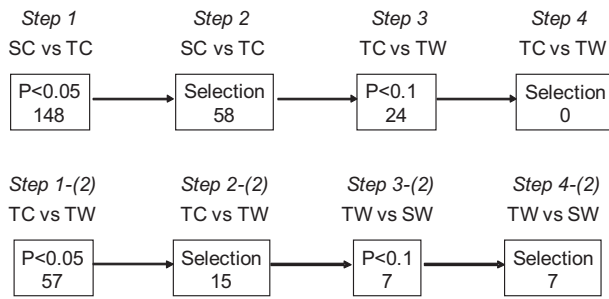


Fig. 1. A scheme of data analysis for protein discovery. Data were analyzed in four steps: step 1, normalized intensities of protein/peptide peaks obtained by SELDI-TOF MS were comprehensively compared between the SC and TC groups; step 2, artifacts were removed from the peaks selected in step 1 and redundant peaks derived from same protein were grouped; step 3, normalized intensities were comprehensively compared between the TW and TC groups in relation to candidates identified in step 1; step 4, peaks with opposite directions than those elicited by training were removed from the peaks selected in step 3. Further, to search for proteins that change in response to exercise training in the whey diet group, but did not change in the other three groups, data were analyzed in four steps: step 1-2, normalized intensities of protein/peptide peaks were comprehensively compared between the TC and TW groups; step 2-2, artifacts were removed and redundant peaks derived from the same protein were grouped; step 3-2, normalized intensities were comprehensively compared between the TW and SW groups in relation to candidates identified in step 1-2; step 4-2, the peaks selected in step 3-2 were checked with regard to the direction of their change. SC, sedentary plus control diet; SW, sedentary plus whey diet; TC, training plus control diet; TW, training plus whey diet.

peaks selected in step 1-2 and redundant peaks derived from the same protein were grouped; step 3-2, normalized intensities of protein/peptide peaks obtained by SELDI-TOF MS were comprehensively compared between the TW and SW groups in relation to candidates identified in step 1-2; step 4-2, peaks selected in step 3-2 were checked with regard to the direction of their change (Fig. 1).

Purification and identification of protein biomarkers

The protein biomarkers were purified as follows. A strong anion-exchange column, Q-sepharose HP (GE Healthcare), was equilibrated with binding buffer (50 mM Tris-HCl [pH 9.0] containing 1 M urea and 0.22% CHAPS). An aliquot (1 mL) of normal mouse plasma (Pel-Freez, Rogers, AR, USA) was diluted 1.5-fold with binding buffer, filtrated with a 0.45- μ m polyvinylidene difluoride membrane, and applied to the Q-sepharose HP column. In total, the column was washed three times with 50 mM Tris-HCl (pH 9.0), with 100 mM sodium acetate (pH 5.0), and with 50 mM sodium citrate (pH 3.0). Final elution was performed with a solution containing 33.3% isopropanol, 16.7% ACN, and 0.1% trifluoroacetic acid and, in the sequence, SELDI-TOF MS analysis was carried out.

The obtained eluate was desalted and concentrated by acetone precipitation and the precipitate was resuspended in a rehydration buffer without reducing agent (7 M urea, 2 M thiourea, 2% CHAPS, and 0.25% ZOOM Focusing Buffer [pH 3–7; Invitrogen, Carlsbad, CA, USA]). Suspensions were subjected to two-dimensional polyacrylamide gel electrophoresis (PAGE) as follows. For the first dimension, IPG dry strips (pH 4–7 non-linear (NL), 7 cm; GE Healthcare) were rehydrated for 13 h with the same solution described above and isoelectric focusing was conducted using an IPGphor II instrument (GE Healthcare). For the second dimension, strips were equilibrated for 30 min in 6 M urea, 30% glycerol, 2% sodium dodecylsulfate, and 50 mM Tris-HCl (pH 8.8) in the absence of a reducing agent and then subjected to a 2-dimensional run (tricine gel, 15–20% gradient; DRC, Tama, Tokyo, Japan). Gels were stained with Coomassie brilliant blue G-250.

To confirm whether the proteins isolated on PAGE gels were target biomarkers, passive elution was performed as follows. Isolated protein bands and spots were excised from PAGE gels and destained with 50% methanol containing 50 mM ammonium bicarbonate. The pieces of gel were then soaked in 50% formic acid, 25% isopropanol, and 15% ACN and shook for at least 2 h to extract proteins. Obtained extracts were applied to the H50 ProteinChip array to measure molecular mass values. After confirming molecular mass values of the extracts, corresponding spots on the replicate gel were analyzed by peptide mass fingerprinting. Procedures for in-gel trypsin digestion and extraction of tryptic peptides for peptide mass fingerprinting were as described by Shevchenko et al. [20]. TOF-MS spectra of tryptic peptides were acquired with a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), and some of the intense

peaks observed in TOF-MS spectra were analyzed in tandem mass spectrometric mode to obtain MS/MS spectra. Obtained TOF-MS and MS/MS spectra were analyzed using mass values of mono-isotopic peaks for searches (MASCOT: <http://www.matrixscience.com>) against the SwissProt database.

Data analysis

Data processing and analysis of the protein profile were performed using Ciphergen Express 3.0 software (Bio-Rad). After baseline subtraction, processed spectra were normalized to the total ion current. Detected peaks were grouped into peak cluster lists and *P* values were calculated using the Mann-Whitney *U* test to compare normalized peak intensities between groups. Although the *P* value is normally set at 0.05 to indicate a statistically significant difference, in this study *P* < 0.1 was accepted as an indicator of a statistically significant difference in the secondary screening for the discovery of biomarker, with the aim of increasing the probability of identifying candidate biomarkers. For the remaining measurements, differences between groups were evaluated by two-way analysis of variance or Student's *t* test. If analysis of variance was significant, the Tukey-Kramer test was used to identify differences between mean values. In the analyses, *P* < 0.05 was considered statistically significant. Results were shown as mean \pm standard error.

Results

Endurance performance and muscle glycogen

The time to exhaustion during running for mice fed the whey diet (63.2 ± 7.0 min) was longer compared with mice fed the control diet (36.6 ± 9.4 min), indicating that intake of whey protein improved aerobic performance (Fig. 2A). Glycogen content in the gastrocnemius muscle was significantly increased from 1.17 ± 0.11 to 1.53 ± 0.16 mg/g by training, and the whey hydrolysate led to a further increase of 2.23 ± 0.25 mg/g (Fig. 2B). In addition, muscle glycogen content showed a tendency to increase in sedentary animals provided the whey diet.

Identification of plasma proteins changed by training plus whey protein hydrolysate

Protein profiling of plasma sample obtained from individuals was performed by SELDI-TOF MS. Consequently, because the protein biomarker whose change was accelerated by whey and by training was not found, we searched for proteins that changed in response to exercise training in the whey diet group, but did not change by training alone. As a result of this analysis, seven specific peaks (mass/ion ratio [m/z] 5280, m/z 6829, m/z 7011, m/z 7924, m/z 13 677, m/z 13 880, and m/z 47 094) were identified as protein biomarker candidates that may change only when exercise training is combined with a whey diet (Table 2).

We attempted to identify the proteins that corresponded to the seven peaks listed above. The isoelectric point of each protein could be assumed according to the detection conditions of the peak (fraction, chromatographic surface chip, and binding buffer). Thus, the fraction that included the target protein was initially separated according to the isoelectric point by ion exchange chromatography and subjected to two-dimensional PAGE (Fig. 3). After PAGE, spots were passively eluted from the gel to confirm that the target protein had been isolated. Because the molecular mass of the target protein was low, the corresponding spot on the replicate gel was analyzed using peptide mass fingerprinting followed by MS/MS. Consequently, the m/z 7924 peak was identified as apolipoprotein (apo-) A-II, which had five tryptic peptides sequenced by tandem mass spectrometry (score = 70 using the MASCOT MS/MS ion search tool, 52% coverage). The m/z 7924 peak corresponds to the molecular mass value calculated from the amino acid sequence of mouse apo-A-II, excepting the signal peptide sequence, nine amino acids of

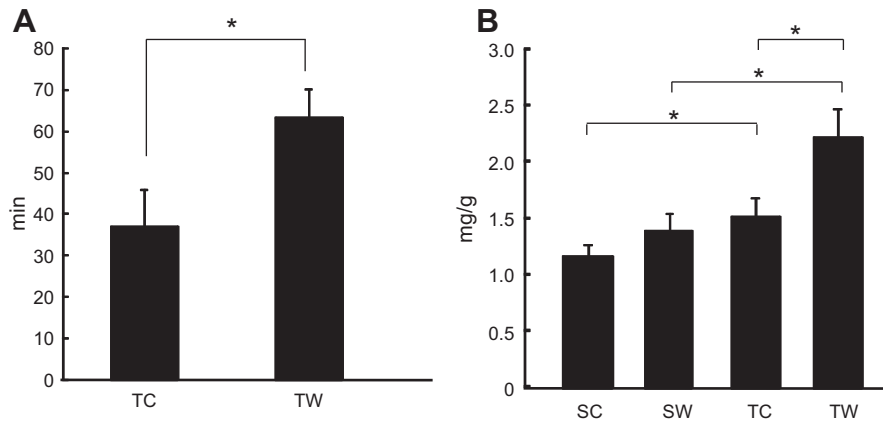


Fig. 2. Exercise performance (A) and muscle glycogen content (B). Values are presented as mean \pm SE. *Significant difference at $P < 0.05$. SC, sedentary plus control diet; SW, sedentary plus whey diet; TC, exercise training plus control diet; TW, exercise training plus whey diet.

the C-terminal, and pyro-glutamylolation of the N-terminal. The peak observed at the m/z 7924 was identified as a truncated isoform of apo-A-II.

Furthermore, with exception of the m/z 5280 peak, we succeeded in identifying the other five peaks. The m/z 7011 peak was identified as apo-C-I; the m/z 6829 peak was identified as a truncated isoform of apo-C-I that lacks N-terminal threonine and proline; m/z 13 677 and 13 880 peaks were identified as transthyretin and sinapine acid plus transthyretin, respectively; the m/z 47 094 peak was identified as β_2 -glycoprotein-1 (β_2 GP1). β_2 GP1 is a glycoprotein abundant in plasma and is known as apo-H. The peptide corresponding to the m/z 5280 peak could not be purified because its concentration in the plasma of mice is very low. In summary, four specific proteins were identified from the seven selected peaks (Fig. 4). With regard to transthyretin, compared with animals provided the control diet, it was not changed in TW and SW animals; this suggests that the change in transthyretin is not specific for the TW group of animals, in which exercise training and intake of whey protein were combined. Therefore, to distinguish it from the other three proteins, transthyretin was excluded from the list of candidate biomarkers.

Discussion

In the present study, we demonstrated that mice fed whey protein in the diet could run for a longer time after exercise

Table 2
Protein/peptide clusters having statistically significant differences in their peak intensities between the TC and TW groups

m/z (Dalton)	Fraction	P	Intensity (mean \pm SE)	
			TC	TW
CM10 low laser energy				
5280.29	4	0.009	0.48 \pm 0.05	0.62 \pm 0.06
7011.11	1	0.003	6.81 \pm 0.54	4.93 \pm 0.38
7924.51	3	0.035	0.69 \pm 0.07	0.82 \pm 0.06
CM10 high laser energy				
13 677.95	1	0.001	0.31 \pm 0.03	0.18 \pm 0.02
13 880.89	1	0.003	0.26 \pm 0.03	0.16 \pm 0.02
47 094.84	1	0.009	0.066 \pm 0.005	0.077 \pm 0.002
Q10 low laser energy				
6829.24	3	0.010	2.84 \pm 0.34	2.65 \pm 0.30

m/z, mass/ion ratio; TC, exercise training plus control diet; TW, exercise training plus whey diet

training compared with mice fed the control casein diet and had higher glycogen levels in the skeletal muscle. The amount of glycogen stored in skeletal muscle is closely related to endurance because it is needed for muscle contraction and thus affects exercise performance. Therefore, the increase of glycogen resynthesis could lead to the promotion of endurance. We used a whey protein hydrolysate (obtained from whey protein treated with proteases) that contains small peptides and does not need to be digested, so rapid absorption can be expected as occurs with free amino acids. Also, some peptides could be beneficial as new functional factors such as amino acids, as indicated in previous studies [5,21]. Such characteristics of whey hydrolysate may accelerate the improvement of glucose resynthesis in the skeletal muscle.

Protein levels in the blood reflect various physiologic and pathologic conditions, and certain proteins have been identified as biomarkers for some conditions. For example, Zhang et al. [11] reported that apo-A-I, a truncated form of transthyretin, and a cleavage fragment of inter- α -trypsin inhibitor heavy-chain H4 were identified as biomarkers in early-stage ovarian cancer using

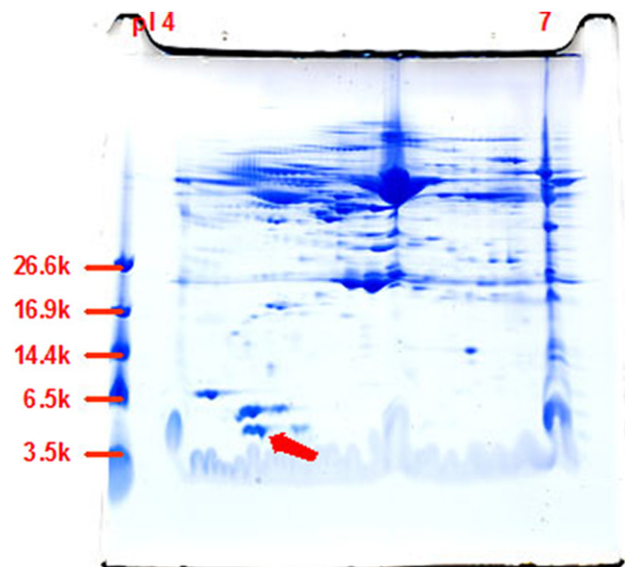


Fig. 3. Isolation of the m/z 7924 protein (arrow) by two-dimensional electrophoresis. pI, isoelectric point.

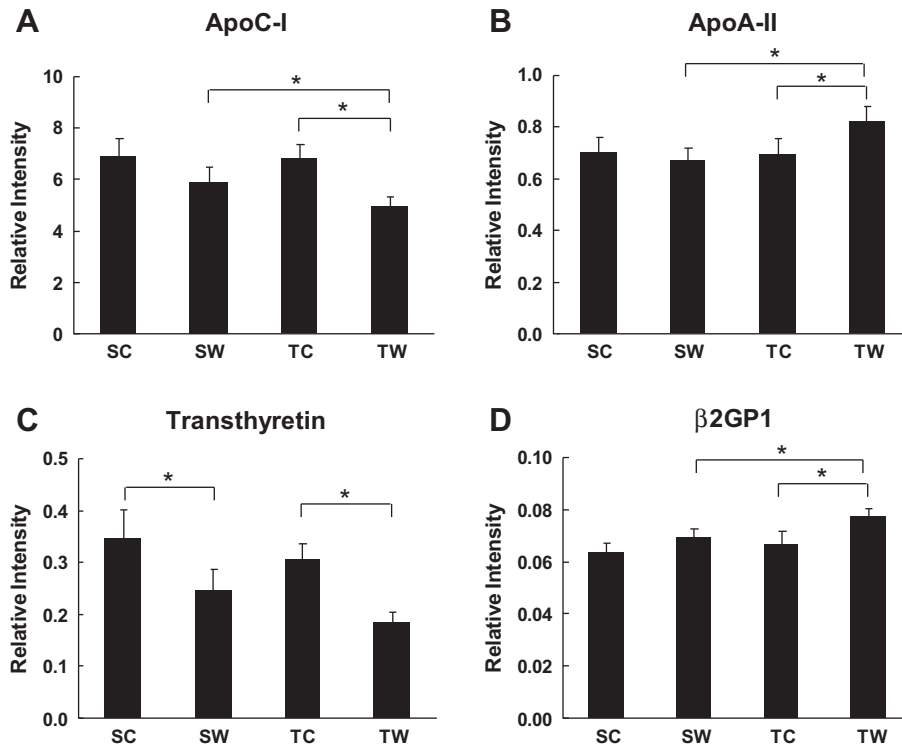


Fig. 4. (A–D) Peak intensity of identified proteins. Values are presented as mean \pm SE. *Significant difference at $P < 0.05$. Apo, apolipoprotein; β 2GP1, β_2 -glycoprotein-1; SC, sedentary plus control diet; SW, sedentary plus whey diet; TC, exercise training plus control diet; TW, exercise training plus whey diet.

the SELDI-TOF MS system. Also, candidate biomarkers that relate to diabetic nephropathy [13,14], atherosclerosis [15], and cardiovascular disease [16] have been identified in human and animal studies. In addition, in the field of nutrition, proteomics investigations have been developed for the mechanistic elucidation of nutrient action [22]. In the presented study we attempted the identification of plasma protein biomarkers for sports science by performing a comprehensive protein profiling analysis using a protein chip. If a blood protein was altered more by intake of whey protein hydrolysate than by training only, it could be a biomarker for endurance and glycogen resynthesis capacity. However, we could not identify such proteins and thus attempted to identify proteins that were changed by training plus an intake of whey protein but were not changed by training or a dietary intake of whey protein alone. Consequently, we identified changes of three proteins (apo-A-II, apo-C-I, and β 2GP1) that have not been reported previously. These three proteins may be useful as biomarkers of the physiologic state, although they would not reflect all conditions because training alone did not cause any changes. That is, the proteins do not necessarily change when endurance improves, but improvement of performance may always be expected when a change of these proteins is found.

Several functions of apo-A-II, apo-C-I, and β 2GP1 that were identified in this study were reported previously. Apo-A-II is an 8.7-kDa protein that is synthesized in the liver and secreted into the plasma; it is the second most abundant protein in high-density lipoprotein, comprising \sim 20% of the total mass of protein in high-density lipoprotein. Although its physiologic function is still unclear, it is believed that a constant level of apo-A-II is essential for maintaining the homeostatic regulation of lipid metabolism [23,24] and for displacing apo-A-I from

high-density lipoprotein particles [25]. Therefore, apo-A-II is associated with protection against cardiovascular diseases by regulation of free fatty acids and triacylglycerol levels in the plasma and hepatic lipase activity [24,26,27]. Apo-C-I is a component of several lipoproteins such as very low-density lipoprotein, intermediate-density lipoprotein, chylomicron, and high-density lipoprotein. It has been suggested that apo-C-I may impair very low-density lipoprotein clearance from the blood by inhibiting the uptake of very low-density lipoprotein particles by the liver [28]. Therefore, apo-C-I may have an important role in the metabolism of nutrients and may be useful for early detection of metabolic abnormalities, as observed in women with polycystic ovary syndrome [29]. Although the function of β 2GP1 is largely unknown, it has recently been shown to have antiangiogenic properties. It has been shown that β 2GP1 inhibits vascular endothelial growth factor- and basic fibroblast growth factor-induced angiogenesis [30] and activates the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells [31]. The changes may contribute to additional glucose uptake and glycogen storage capacity. Thus, it would be interesting in the future to clarify the effects of these proteins on muscle glucose metabolism. Conversely, it should be also considered that the variation of these proteins by whey may occur as a result of performance improvement, which could alter relative exercise intensity. In addition, whey is rapidly absorbed after intake and transiently elevates amino acids in the blood compared with casein [32], which may also affect these results. In any event, the beneficial effects related to these proteins were significantly generated by the combination of whey hydrolysate and exercise only, and not by training or whey alone. Our observations suggest that dietary supplementation during training can lead to new outcomes that cannot be obtained by

training or diet alone, besides augmenting the beneficial effects of exercise.

Conclusion

We found that dietary intake of whey protein hydrolysate with training improves endurance, in addition to an increase of muscle glycogen resynthesis. Apo-A-II, apo-C-I, and β 2GP1 were altered by whey protein hydrolysate intake with training, but not by whey intake or training alone, according to comprehensive proteomics analysis. These proteins may have potential as biomarkers of endurance and glycogen resynthesis capacity and be part of the mechanism leading to the benefit of whey protein hydrolysate.

References

- [1] de Wit JN. Nutritional and functional characteristics of whey proteins in food products. *J Dairy Sci* 1998;81:597–608.
- [2] Hayes A, Cribb PJ. Effect of whey protein isolate on strength, body composition and muscle hypertrophy during resistance training. *Curr Opin Clin Nutr Metab Care* 2008;11:40–4.
- [3] Dangin M, Guillet C, Garcia-Rodenas C, Gachon P, Bouteloup-Demange C, Reiffers-Magnani K, et al. The rate of protein digestion affects protein gain differently during aging in humans. *J Physiol* 2003;549:635–44.
- [4] Hall WL, Millward DJ, Long SJ, Morgan LM. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *Br J Nutr* 2003;89:239–48.
- [5] Betts JA, Williams C, Boobis L, Tsintzas K. Increased carbohydrate oxidation after ingesting carbohydrate with added protein. *Med Sci Sports Exerc* 2008;40:903–12.
- [6] Morifuji M, Sakai K, Sanbongi C, Sugiura K. Dietary whey protein increases liver and skeletal muscle glycogen levels in exercise-trained rats. *Br J Nutr* 2005;93:439–45.
- [7] Morifuji M, Sakai K, Sugiura K. Dietary whey protein modulates liver glycogen level and glycoregulatory enzyme activities in exercise-trained rats. *Exp Biol Med* 2005;230:23–30.
- [8] Saito S, Yoshitake Y, Suzuki M. Enhanced glycogen repletion in liver and skeletal muscle with citrate orally fed after exhaustive treadmill running and swimming. *J Nutr Sci Vitaminol* 1983;29:45–52.
- [9] Ivy JL, Goforth HW Jr, Damon BM, McCauley TR, Parsons EC, Price TB. Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *J Appl Physiol* 2002;93:1337–44.
- [10] van Hall G, Saris WH, van de Schoor PA, Wagenmakers AJ. The effect of free glutamine and peptide ingestion on the rate of muscle glycogen resynthesis in man. *Int J Sports Med* 2000;21:25–30.
- [11] Zhang Z, Bast RC Jr, Yu Y, Li J, Sokoll LJ, Rai AJ, et al. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004;64:5882–90.
- [12] Engwegen JY, Mehra N, Haanen JB, Bonfrer JM, Schellens JH, Voest EE, et al. Validation of SELDI-TOF MS serum protein profiles for renal cell carcinoma in new populations. *Lab Invest* 2007;87:161–72.
- [13] Kim HJ, Cho EH, Yoo JH, Kim PK, Shin JS, Kim MR, et al. Proteome analysis of serum from type 2 diabetics with nephropathy. *J Proteome Res* 2007;6:735–43.
- [14] Merchant ML, Klein JB. Proteomics and diabetic nephropathy. *Semin Nephrol* 2007;27:627–36.
- [15] Dursun E, Ozben B, Monari E, Cuoghi A, Tomasi A, Ozben T. Proteomic profiling in apolipoprotein E-deficient mice during atherosclerosis progression. *Acta Histochem* 2010;112:178–88.
- [16] Anderson L. Candidate-based proteomics in the search for biomarkers of cardiovascular disease. *J Physiol* 2005;563:23–60.
- [17] Billat VL, Mouisset E, Roblot N, Melki J. Inter- and intrastain variation in mouse critical running speed. *J Appl Physiol* 2005;98:1258–63.
- [18] Kemi OJ, Loennechen JP, Wisløff U, Ellingsen Ø. Intensity-controlled treadmill running in mice: cardiac and skeletal muscle hypertrophy. *J Appl Physiol* 2002;93:1301–9.
- [19] Passonneau JV, Lauderdale VR. A comparison of three methods of glycogen measurement in tissues. *Anal Biochem* 1974;60:405–12.
- [20] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996;68:850–8.
- [21] Morifuji M, Koga J, Kawanaka K, Higuchi M. Branched-chain amino acid-containing dipeptides, identified from whey protein hydrolysates, stimulate glucose uptake rate in L6 myotubes and isolated skeletal muscles. *J Nutr Sci Vitaminol* 2009;55:81–6.
- [22] Kussmann M, Affolter M. Proteomics at the center of nutrigenomics: comprehensive molecular understanding of dietary health effects. *Nutrition* 2009;25:1085–93.
- [23] Blanco-Vaca F, Escolà-Gil JC, Martín-Campos JM, Julve J. Role of apoA-II in lipid metabolism and atherosclerosis: advances in the study of an enigmatic protein. *J Lipid Res* 2001;42:1727–39.
- [24] Tailleux A, Duriez P, Fruchart JC, Clavey V. Apolipoprotein A-II, HDL metabolism and atherosclerosis. *Atherosclerosis* 2002;164:1–13.
- [25] Durbin DM, Jonas A. The effect of apolipoprotein A-II on the structure and function of apolipoprotein A-I in a homogeneous reconstituted high density lipoprotein particle. *J Biol Chem* 1997;272:31333–9.
- [26] Boucher J, Ramsamy TA, Braschi S, Sahoo D, Neville TA, Sparks DL. Apolipoprotein A-II regulates HDL stability and affects hepatic lipase association and activity. *J Lipid Res* 2004;45:849–58.
- [27] Julve J, Escolà-Gil JC, Rotllan N, Fiévet C, Vallez E, de la Torre C, et al. Human apolipoprotein A-II determines plasma triglycerides by regulating lipoprotein lipase activity and high-density lipoprotein proteome. *Arterioscler Thromb Vasc Biol* 2010;30:232–8.
- [28] Jong MC, Hofker MH, Havekes LM. Role of apoCs in lipoprotein metabolism. Functional differences between apoC1, apoC2, and apoC3. *Arterioscler Thromb Vasc Biol* 1999;19:472–84.
- [29] Huang S, Qiao J, Li R, Wang L, Li M. Can serum apolipoprotein C-I demonstrate metabolic abnormality early in women with polycystic ovary syndrome? *Fertil Steril* 2010;94:205–10.
- [30] Yu P, Passam FH, Yu DM, Denyer G, Krilis SA. Beta2-glycoprotein I inhibits vascular endothelial growth factor and basic fibroblast growth factor induced angiogenesis through its amino terminal domain. *J Thromb Haemost* 2008;6:1215–23.
- [31] Sakai T, Balasubramanian K, Maiti S, Halder JB, Schroit AJ. Plasmin-cleaved beta-2-glycoprotein I is an inhibitor of angiogenesis. *Am J Pathol* 2007;171:1659–69.
- [32] Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL, Beaufrère B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci U S A* 1997;94:14930–5.