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Research Article

Distinct Effects of Dietary Whey Peptide and Soy Protein on Denervation-Mediated Muscle Atrophy

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Abstract

Purpose: We examined whether various diet proteins, such as soy protein, whey protein and whey peptides, affected denervation-mediated muscle atrophy.

Methods: Six-week-old C57/BL6J male mice consumed test diets that contained casein, soy protein, whey peptide, or whey protein for 2 weeks. One hind limb of mouse was enervated on Day 0. Hind limb skeletal muscles were dissected and weighed on Day 4 or Day6. Protein and transcripts expressed in these muscles were analyzed.

Results: Denervation caused reductions in soleus and tibialis anterior muscle wet weights. In the soleus muscle, this effect was significantly prevented with the whey peptide on Day 6, but not the soy and whey protein diets, compared to the case in diet. In the tibialis anterior muscle, the effect was prevented with the soy protein, but not the whey protein or peptide diet, compared to the casein diet. Denervation up regulated the expression of muscle atrophy-related genes, MAFbx/atrogin-1 and MuRF-1. In the soleus muscle, the whey peptide diet significantly suppressed MuRF-1 upregulation on Day 6, while the soy protein, but not the whey protein or peptide diet, significantly suppressed MAFbx/atrogin-1 upregulation in the tibialis anterior on Day 4, compared to the case in diet. The mechanism was elucidated by examining Akt and S6 kinase (S6K) phosphorylation in gastrocnemius muscle at 4 days after denervation. Muscle denervation significantly inhibited both Akt and S6K phosphorylation, compared to non-denervated muscle. The effect on Akt was significantly prevented with the whey peptide, soy protein, and whey protein diets, compared to the case in diet. S6K phosphorylation was significantly increased with the whey peptide diet, in both non-denervated and denervated gastrocnemius muscle. In contrast, the soy and whey protein diets had little effect on S6K phosphorylation compared to the case in diet.

Conclusion: The present study showed that whey peptide was most effective in preventing denervation-mediated atrophy of the slow-twitch type muscle, while soy protein preferentially suppressed the atrophy of the first-twitch type muscle.

Keywords: Denervation; Mice; Muscle atrophy; Soy protein; Whey protein or peptide

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Abbreviation

Atrogene: muscle atrophy-associated ubiquitin ligase; BCAA: Branched-Chain Amino Acids; Cbl-b: Casitas B-cell lymphoma-b; Cblin peptide: Cbl-b inhibitory peptide; GA: Gastrocnemius muscle; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; IGF-1: Insulin-like Growth Factor-1; IRS-1: Insulin Receptor Substrate-1; MAFbx/atrogin-1: Muscle Atrophy F-box protein/atrogin-1; mTOR: mammalian Target of Rapamycin; MuRF-1: Muscle RING Finger protein-1; qRT-PCR: quantitative Reverse Transcription Polymerase Chain Reaction; S6K: S6 kinase; TA: Tibialis Anterior; TKB domain: Tyrosine Kinase Binding domain; whey peptide: whey protein-derived peptides.

Introduction

Skeletal muscle mass is controlled by the balance between protein synthesis and degradation. Muscle atrophy is caused by both increasing protein degradation and decreasing protein synthesis [1]. Insulin-like growth factor-1 (IGF-1) signaling is a major pathway of skeletal muscle growth. In our previous studies, we showed that unloading stress caused over expression of the ubiquitin ligase, Casitas B-cell

lymphoma-b (Cbl-b). This over expression enhanced the ubiquitination and degradation of insulin receptor substrate-1(IRS-1) [2,3], which suggested that Cbl-b was a muscle atrophy-associated ubiquity in ligase (atrogene). Indeed, the Cbl-b-mediated loss of IRS-1 induced the expression of other atrogenes, such as muscle atrophy F-box protein (MAFbx)/atrogin-1 and muscle RING finger protein-1 (MuRF-1) [4,5], which contributed to muscle atrophy [3].

To prevent unloading-mediated muscle atrophy, we developed a Cbl-b inhibitory peptide (called Cblin peptide) with the amino acid sequence, DGpYMP (pY is a phosphorylated tyrosine). This Cblin peptide interacted with the Tyrosine Kinase Binding (TKB) domain of Cbl-b to prevent Cbl-b-mediated IRS-1 ubiquitination [6]. Consequently, injections of Cblin peptide into muscle significantly prevented denervation-mediated muscle atrophy in mice [4]. Furthermore, the soy protein, 11S glycinin, contains a Cblin-like sequence, DIYNP, which was shown to inhibit Cbl-b-mediated ubiquitination and degradation of IRS-1 [7]. Dietary soy glycinin also prevented denervation-induced muscle atrophy in mice [7]. In addition, some dietary proteins are important sources of Branched-Chain Amino Acids (BCAA), which specifically regulate muscle protein synthesis [8,9]. For example, dietary whey protein was identified as a beneficial protein that prevented muscle atrophy in animals and humans [10-13], because it contained a higher proportion of BCAAs, particularly leucine, compared toother dietary proteins. BCAAs, particularly leucine, activate the mammalian Target of Rapamycin (mTOR) to stimulate muscle protein synthesis [10]. Thus, amino acids and/or peptides derived from dietary proteins can regulate protein metabolism in skeletal muscle.

Bed-ridden elderly population continues increasing remarkably for a super aging society in Japan. To conquer this social problem, we focused on the regulatory function of amino acids and/or peptides on muscle protein metabolism. In this study, we examined the inhibitory effects of various dietary proteins/peptides, including whey protein, whey protein-derived peptides (whey peptide), soy protein, and casein, to determine which dietary protein(s) could best protect against muscle atrophy caused by unloading. The results suggest that amino acids or peptides derived from whey and soy proteins are available for the functional foods against muscle atrophy.

Materials and Methods

Diets and animal model

After one week of adaptation, we started giving 6-week-old C57/BL6J male mice (Japan SLC, Shizuoka, Japan) protein-specific AIN-93G-based diets. The nutritional composition of each diet is shown in Table1. Each diet contained one of the following four proteins: case in (milk casein, Oriental Yeast Co.,), whey protein (TATUA 902, TATUA Co-operative Dairy), whey peptide (HW-3, Megmilk Snow Brand Co., Ltd., Japan), or soy protein (a kind gift from Fuji Oil Co., Osaka, Japan). Each 100 g diet contained 17.3 g protein. The mice in each dietary protein group consumed *ad libitum*. After consuming the diets for 2 weeks, mice were denervated; then, they continued consuming the diets until sacrifice.

The denervated mouse model (performed after 2 weeks feeding) was prepared by resecting the sciatic nerve in the left hind limb of each mouse on Day 0. Mice were sacrificed on Day 4 or Day 6 after denervation, and both denervated and non-denervated hind limb skeletal muscles were isolated from a mouse for analysis.

Diet / Contents	Casein (g/100g)	Soy (g/100g)	Whey protein (g/100g)	Whey peptide (g/100g)
Casein	20.0	0.0	0.0	0.0
Soy protein	0.0	19.8	0.0	0.0
Whey protein	0.0	0.0	0.0	21.9
Whey peptide	0.0	0.0	22.4	0.0
L-Cysteine	0.3	0.3	0.3	0.3
β-Starch	39.9	39.9	39.9	39.9
α-Starch	13.2	13.3	13.2	13.2
Lactose hydrate	2.5	2.5	1.0	0.0
Sucrose	7.7	7.7	7.7	7.7
Soybean oil	7.0	7.1	5.8	7.2
Cellulose	5.0	5.0	5.0	5.0
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1.0	1.0	1.0	1.0
KH_2PO_4	0.0	0.0	0.2	0.4
t-Butylhydroquinone	0.0014	0.0014	0.0014	0.0014

Table 1: Composition of the four experimental diets.

Diets were designed to provide similar amounts of nitrogen in protein and all other nutrients.

All protocols in this study were conducted according to the Guide for the Care and Use of Laboratory Animals at the University of Tokushima. All protocols were approved by the Committee for the Care and Use of Laboratory Animals at the University of Tokushima.

Immunoblotting

Immunoblotting analyses were performed as described previously [14]. We used the following antibodies: anti-phosphorylated Akt antibody (Cell signaling), anti-Akt antibody (Cell signaling), anti-phosphorylated S6 kinase (S6K; Cell signaling), anti-S6K (Santa Cruz Biotechnology), and anti-GAPDH antibody (Santa Cruz Biotechnology).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was performed with an ABI 7300 Real Time PCR System (Applied Biosystem, Foster City, CA) using SYBR™ Green dye (Applied Biosystem) as described previously [3]. The primer sequences are listed in Table 2.

Statistical analysis

All data are expressed as the mean ± standard deviation of 6 individual samples per group. Differences between groups were analyzed via a two-way analysis of variance. Analyses were performed with SPSS (release 6.1, SPSS Japan, Tokyo, Japan). Statistical significance was tested with the Bonferroni's test, and P-values < 0.05 were considered significant.

Results

Intakes of food, energy, and protein in each diet group

We found no significant difference in daily intakes of food, energy, or protein among the four groups of mice (Table 3).

Target gene		Sequence	Length (bp)
Cbl-b	S	5'-GAGCCTCGCAGGACTATGAC-3'	241
	AS	5'-CTGGCCACTTCCACGTTATT-3'	
GAPDH	S	5'-ACCCAGAAGACTGTGGATGG-3'	125
	AS	5'-TTCAGCTCTGGGATGACCTT-3'	
MAFbx/atrogin-1	S	5'-GGCGGACGGCTGGAA-3'	100
	AS	5'-CAGATTCTCCTTACTGTATACCTCCTTGT-3'	
MuRF-1	S	5'-ACGAGAAGAAGAGCGAGCTG-3'	179
	AS	5'-CTTGGCACTTGAGAGGAAGG-3'`	

Table 2: Primer sequences for quantitative real-time PCR.

AS: Antisense Primer; S: Sense primer; Cbl-b: Casitas B-cell lymphoma-b (ubiquitin kinase); MAFbx/atrogin-1: Muscle Atrophy F-box protein; MuRF-1: Muscle RING Finger protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Diet / Intakes	Casein	Soy	Whey protein	Whey peptide
Food intake(g/day)	3.42±0.01	3.55±0.09	3.23±0.09	3.15±0.27
Energy intake(kcal/day)	13.25±0.04	13.7±0.37	12.51±0.36	12.21±0.25
Protein intake (g/day)	0.65±0.13	0.61±0.02	0.55±0.02	0.54±0.12

Table 3: Daily intake of food, energy, and protein in each diet group.

Intakes were measured as the difference in weight (g) between the foods supplied minus the food remaining in the feeder each day. Energy was calculated as the sum of kcal/g calculated for each nutrient.

Whey peptide inhibition of denervation-induced reductions in muscle wet weight

Denervation caused significant reductions in the wet weights of all hind limb muscles, including the soleus, gastrocnemius, and Tibialis Anterior (TA) muscles, measured on days 4 and 6 (Figure 1). The denervation-induced reduction in soleus muscle wet weight observed with the casein diet on Day 6 was significantly prevented with the whey peptide diet, but the soy and whey protein diets did not have significant effects (Figure 1A). The denervation-induced reduction in TA wet weight observed with the casein diet on Day 6 was significantly suppressed with the soy protein diet, but not with the whey protein or whey peptide diets. Also, none of these inhibitory effects achieved significance on Day 4 (Figure 1). In contrast, the reduction in gastrocnemius muscle wet weight observed with the casein diet was somewhat (but not significantly) suppressed with the soy protein diet, whey protein, and whey peptide diet son Day 4, but not with all tested diet son Day 6 (Figure 1C).

Dietary protein inhibition of atrogenes expression

The effects of whey peptide and soy protein diet son the 6-day denervation-induced reductions in soleus and TA muscle wet weights prompted us to examine the expression of atrogenes. Specifically, we investigated MAFbx/atrogin-1, MuRF-1, and Cbl-b expression in the soleus and TA muscles of mice fed the test diets. Consistent with previous reports [7,15], we found that denervation significantly increased the expression of such muscle atrophy-associated ubiquitin ligases, in both TA and soleus muscles (Figure 2). Compared to the casein diet, the whey peptide diet significantly suppressed denervation-induced expression of MuRF-1 in soleus muscle on Day 6, whereas the soy and whey protein diets did not affect expression of MAFbx/atrogin-1 or MuRF-1 in soleus muscle (Figure 2A). In contrast, the soy protein diet significantly inhibited the expression of MAFbx/atrogin-1 in TA muscle on Day 4 after denervation, but the whey protein/peptide diets

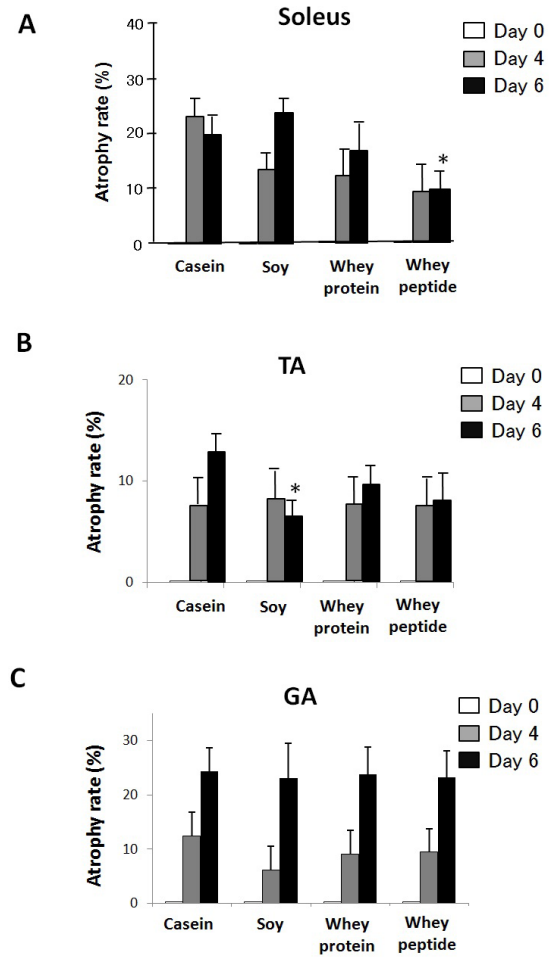

Figure 1: Effect of whey peptide on muscle wet weights.

C57BL/6 mice were divided into the following four groups (n =6/group): casein diet, soy diet, whey protein diet, and whey peptide diet. After 2weeks on each diet, sciatic nerve resections were performed. On Day 0 (just before denervation) or on Day 4 and Day 6 days after denervation, denervated or non-denervated hind limb skeletal muscles were isolated and weighed. Denervation-induced atrophy rates are shown for (A) soleus muscles; (B) Tibialis Anterior muscles (TA); and (C) Gastrocnemius muscles (GA) from mice in each diet group. The rate of atrophy (% reduction) was defined as the mean wet weight of denervated muscle (day 4 or day 6) compared to the mean wet weight of non-denervated muscle (Day 0). Data are the mean ± SD. *P< 0.05 versus the casein diet on day 6 after denervation, evaluated with Bonferroni's test.

failed to inhibit MAFbx/atrogin-1expression significantly, compared to the casein diet (Figure 2B). None of the tested protein diets affected the denervation-induced up regulation of MuRF-1expression in TA muscle, compared to the casein diet. Also, none of the tested diets significantly affected Cbl-b expression in either soleus or TA muscle on Day 4 and Day 6.

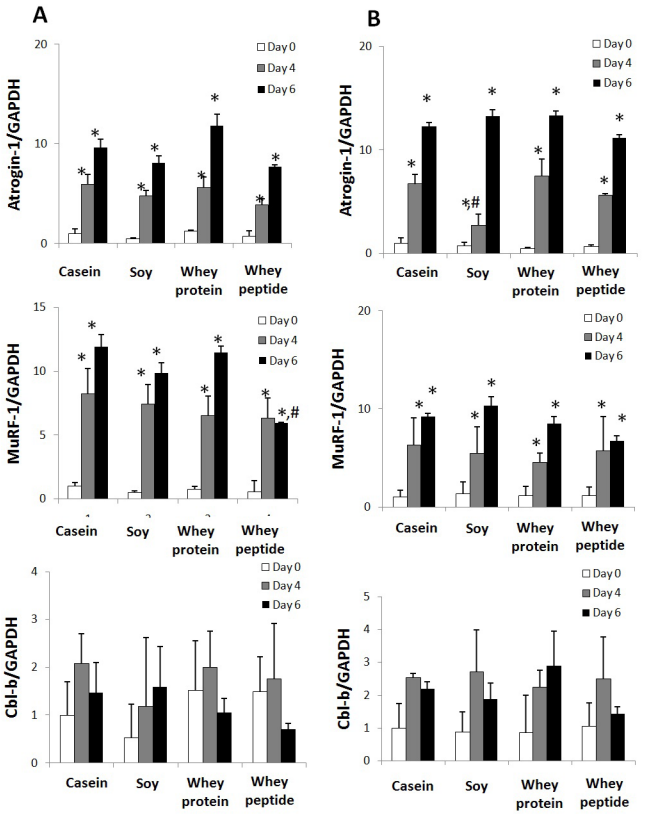

Figure 2: Inhibitory effect of dietary proteins on muscle atrophy-related genes.

C57BL/6 mice were divided into the following four groups: (n =6/group): casein diet, soy diet, whey protein diet, and whey peptide diet. On Day 0 (just before denervation) or on Day 4and Day 6after denervation, hind limb skeletal muscles were isolated, RNA was extracted, and reverse-transcribed DNA was prepared for quantitative real-time PCR analyses. For each dietary group, the relative expression levels (compared to GAPDH internal control) of MAFbx/atrogin-1, MuRF-1, and Cbl-b are shown for (A) soleus muscle and (B) tibialis anterior muscle. Data are means ± standard deviations. *P< 0.05 compared to day 0 within each group; #P< 0.05 compared to the casein diet group on the same days, evaluated with the Bonferroni's test.

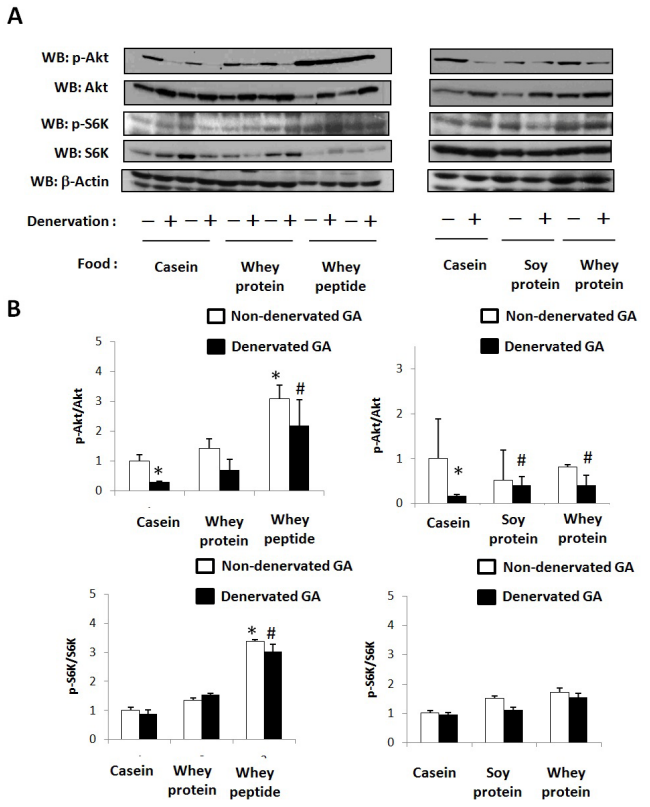

Figure 3: Effects of dietary protein/peptide on the phosphorylation of Akt and S6K in muscle.

(A)C57BL/6 mice were divided into the following four groups (N=6/group): casein diet, soy diet, whey protein diet, and whey peptide diet. After 2weeks on each diet, sciatic nerve resections were performed. On Day 4 after denervation, Gastrocnemius muscles (GA) were isolated, protein lysates were prepared, and Western Blots (WB) was performed. Western blots for the casein and whey protein diet groups were repeatedly performed, because we failed to perform WB for all diet groups on the same membrane. Specific antibody probes were used to determine the amounts of (A) total and phosphorylated Akt (Akt and pAkt, respectively), and total and phosphorylated S6K (S6K and pS6K, respectively). (B) The densitometric analysis was performed [7]. The graph showed the mean ± SD of p-Akt/Akt and p-S6K/S6K for each dietary group. The values of p-Akt/b-act in or p-S6K/b-act in were similar for those of p-Akt/Akt and p-S6K/S6K, respectively (data not shown). *P< 0.05 compared to non-denervated limbs in the casein diet group; #P< 0.05 compared to denervated limbs in the casein diet group; significance was evaluated with the Bonferroni's test.

Effects of dietary protein/peptide on Akt and S6Kphosphorylation in muscle

To elucidate the mechanism underlying the inhibitory effects of dietary proteins, we performed immunoblotting to examine the phosphorylation of Akt and S6 kinase (S6K) in gastrocnemius muscle, at 4 days after denervation. Gastrocnemius muscles were selected for this analysis, because they contain both fast and slow twitch muscle fibers. In addition, the soy protein, whey protein and whey peptide tended to suppress the loss of wet weights in gastrocnemius muscle observed with casein diet (Figure 1C).

In mice fed the casein diet, denervation for 4 days significantly decreased the amount of phosphorylated Akt in gastrocnemius muscle, compared to the amount in non-denervated muscles (Figure 3A). The whey peptide diet significantly increased the amounts of phosphorylated Akt in both non-denervated and denervated gastrocnemius muscles. Although the soy and whey protein diets only slightly affected the amount of total Akt in non-denervated and denervated muscles, they significantly protected the gastrocnemius muscle from denervation-induced dephosphorylation of Akt, compared to the casein diet.

Denervation for 4 days did not change phosphorylation of S6K in gastrocnemius muscle in mice fed the casein diet (Figure 3B). Interestingly, the whey peptide diet significantly increased the amounts of phosphorylated S6K, both in non-denervated and denervated gastrocnemius muscles. However, soy and whey protein diets only slightly affected denervation-induced dephosphorylation of S6K in gastrocnemius muscle, compared to the casein diet.

Discussion

In this study, we examined whether various dietary proteins were effective in preventing denervation-mediated muscle atrophy in mice. We composed diets with the following protein sources: casein, soy protein, whey protein, and whey peptide. We found that soy protein and whey peptide had distinct effects on denervated hind limb skeletal muscles.

Muscle fibers are classified as slow-twitch (type 1oxidative) and fast-twitch (type 2 glycolytic) fibers. We found that, in TA muscle,

which is classified as a fast-twitch muscle fiber, the soy protein diet significantly prevented denervation-induced atrophy. In soleus muscle, which is classified as a slow twitch muscle fiber, the whey peptide diet inhibited denervation-induced atrophy. Previously, we reported that soy glycinin prevented denervation-induced atrophy in skeletal muscle, particularly in TA muscle, in mice [7]. Consistent with that report, the present study showed that the effect of the soy protein diet was strongest on TA muscle, compared to other hind limb skeletal muscles. We previously reported that soy glycinin contained a short sequence that inhibited Cbl-b-mediated IRS-1 ubiquitination [7]. Thus, soy glycinin suppressed denervation-induced gene expression of the muscle atrogene, MAFbx-1/atrogin-1, by protectingIGF-1 signaling [7]. In the present study, we found that the soy protein diet prevented the denervation-induced decrease in phosphorylated Akt in gastrocnemius muscle, of which wet weight was not significantly affected with the soy protein diet. This finding was consist with our previous result that the decrease in Akt-1 phosphorylation was smaller in the denervated TA muscle of the soy glycinin protein group than in the control group [7]. Therefore, these findings suggested that the soy protein diet preferentially have prevented muscle atrophy of the fast-twitch type muscle, including TA muscle, through inhibiting Cbl-b activity.

In contrast, whey peptide diet inhibited denervation-induced muscle weight loss more dramatically in slow-twitch fibers than in fast-twitch fibers. It was previously reported that BCAAs prevented hind limb suspension-induced atrophy in the soleus muscle [15,16]. Yamamoto et al. [17] also reported that BCAAs prevented dexamethasone-induced soleus muscle atrophy. Both whey protein and whey peptide are rich in BCAAs, particularly leucine [18], and their beneficial effects were associated with their high BCAA content [17]. Moreover, leucine promoted muscle protein synthesis by activating the PI3K/Akt/mTOR signaling pathway [11,19].Consistent with that finding, we found that the whey peptide diet significantly stimulated the expression and phosphorylation of S6K, a downstream target of mTOR, in the protein synthesis pathway, in gastrocnemius muscle where the reduction in its muscle wet weight observed with the casein diet was somewhat (but not significantly) suppressed with whey peptide diets on Day 4. Whey peptide may preferentially prevent the denervation-mediated loss of soleus muscle, since BCAA stimulated protein synthesis in type 2a myofibers among fast-twitch fibers [17], and soleus muscle contains type 2a myofibers more than TA muscle [17]. However, several lines of investigations reported that leucine or leucine metabolite had an inhibitory effect on fast-twitch muscle atrophy [20-22]. At present, we cannot explain this discrepancy between those studies and the present study. Further investigation is necessary to elucidate this issue.

Similar to soy protein, the whey peptide inhibited the denervation-induced disturbances inIGF-1 signaling. Jang et al. [16] reported that BCAAs prevented muscle atrophy by suppressing the expression of MAFbx/Atrogin-1 and MuRF-1 [15]. Consistent with that report, we found that the whey peptide diet significantly prevented denervation-induced expression of MuRF-1 in soleus muscle. The whey diet also increased the amounts of Akt and phosphorylated Akt in gastrocnemius muscle. Based on these findings, we suggest that the whey peptide prevented muscle atrophy by suppressing the expression of atrogenes and by activating muscle protein synthesis.

Our results showed that the whey peptide diet significantly inhibited denervation-induced muscle atrophy, but the whey protein diet had little effect on muscle atrophy. Because peptides and amino acids do not require extensive digestion, they are absorbed into the blood more readily than protein [23]. Indeed, blood amino acid concentrations rose faster after ingesting whey peptide than after ingesting whey protein [24]. In addition, the inhibitory effect of whey peptide on fat accumulation was greater than whey protein [25]. Thus, the different absorption efficiencies of whey protein and whey peptide may explain their distinct effects on muscle atrophy.

In conclusion, the present study showed that whey peptide was most effective in preventing denervation-mediated atrophy of the slow-twitch type muscle, while soy protein preferentially suppressed the atrophy of the first-twitch type muscle.

Acknowledgments

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