A Diet Including Red Bell Pepper Juice and Soy Protein Suppress Physiological Markers of Muscle Atrophy in Mice

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Summary Although muscle atrophy can be caused by disuse and lifestyle-related syndromes, it may be possible to prevent this condition through dietary intervention. We hypothesized that a diet including red bell pepper juice (RBPJ) and soy protein isolate (SPI) would prevent muscle atrophy. Accordingly, an experimental diet containing RBPJ and/or SPI was administered for 18 d to normal C57BL/6J mice. The control group was administered a casein diet. Four days before the end of the test period, denervation-induced muscle atrophy and/or sham operation were performed. Anterior tibialis muscle samples were then obtained to assess muscle degradation and perform metabolome analysis. Under the denervation condition, the 20% SPI diet did not alter the mRNA expression levels of muscle atrophy marker genes compared with the 20% casein group. Although the diet comprising RBPJ and 20% casein did not prevent muscle atrophy compared with the control group, the diet containing RBPJ and 20% SPI did. Metabolome analysis revealed that a diet including RBPJ and SPI induced a greater than 1.5-fold change in the levels of 20 muscle atrophy-related metabolites. In particular, the level of S-adenosylmethionine, which concerned with energy metabolism and lifespan, showed a strong positive correlation with the muscle atrophy marker. These findings suggest that a diet including RBPJ and soy protein suppress gene expressions related with muscle atrophy. Further research in humans is needed to confirm whether a combination of RBPJ and SPI can indeed prevent muscle atrophy.

Key Words muscle atrophy, red bell pepper, soy protein, S-adenosylmethionine, metabolome analysis, metabolome

Muscles are categorized based on their structures and functions. They are distributed throughout the body in mammals, including humans, and play a key role in energy expenditure, glucose uptake and metabolism, and physical kinetics. Muscle mass is balanced by protein synthesis and protein breakdown. Muscle atrophy is a symptom of locomotive syndromes triggered by several factors, including aging (1–3). Although some foods are believed to improve muscle atrophy, few studies have examined how dietary ingredients influence muscle decomposition (4).

Red bell pepper (Capsicum annuum L. [RBP]) is a vegetable commonly eaten worldwide and cultivated in various regions, including southern Europe and Japan. We recently produced a bitterless RBP juice (RBPJ) containing a small amount of quercetin-3-O-rhamnoside (5). The advantage of the debittering process developed in our laboratory is that it can specifically remove bitter components without altering the basic nutritional balance. In particular, RBPJ contains high levels of vitamin B6 among its minor constituents. A Maastricht Sarcopenia Study showed that sarcopenic older adults have 10–18% lower intake of five nutrients—n-3 fatty acids, vitamin B6, folic acid, vitamin E, and magnesium—compared with non-sarcopenic older adults (6); vitamin B6 is associated with muscle atrophy. RBPJ intake may have beneficial effects on biological function, particularly muscle tissue; however, to date no detailed investigations have been conducted.

Soy protein isolate (SPI), a major source of dietary protein, comprises three main fractions: lipophilic proteins, β-conglycinin, and glycinin (7). Glycinin is a major storage protein accounting for approximately 40% of the proteins in SPI. SPI has various physiological properties, such as hypcholesterolemic effects (8), blood triglyceride-lowering effects (9, 10), and obesity prevention (11). Abe et al. investigated the ability of dietary ingredients to prevent muscle atrophy following surgical sciatic nerve resection and found that consumption of a 20% glycinin diet improved muscle atrophy compared with animal protein (12). Although glycinin improves muscle atrophy, it must be consumed in...
very large amounts when ingested in the form of SPI. Therefore, a coexisting component that enhances the muscle atrophy-ameliorating effects of glycinin or SPI is required.

In this study, we examined whether simultaneous ingestion of RBPJ and SPI enhanced their respective beneficial effects on muscle atrophy. Interestingly, a diet including both RBPJ and SPI significantly suppressed gene expressions related with denervation-derived muscle atrophy, whereas a casein diet did not.

**MATERIALS AND METHODS**

**Animals, denervation, and diets.** Five-week-old male C57BL/6J mice (CLEA Japan, Tokyo, Japan) were housed in individually ventilated cages (IVC Mouse Racks; Innovative, San Diego, CA) under controlled conditions (temperature, 23±1°C; humidity, 55±5%; 7:00–19:00 light cycle). The mice were fed commercial chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) for 5 d prior to the experimental study (n=8/group).

Sciatic nerve resection inducing muscle atrophy was performed as described by Abe et al. (12) with some modifications. Briefly, under isoflurane anesthesia, 2 mm of skin was incised, the sciatic nerve of the right leg was cut, a 10-mm section of the nerve was excised, and the skin was sutured. In the sham operation, 2 mm of skin was incised and the skin was sutured without excision of the sciatic nerve of the right leg as a control. No inflammation was observed at the incision site on the day after surgery. Experimental diet compositions were based on the AIN-93G formula (13) with some modifications. The compositions of the experimental diets are shown in Table 1. Vitamin-free casein (Oriental Yeast Co., Ltd.) and SPI (Fujipro; Fuji Oil Co., Ltd., Osaka, Japan) were provided as sources of dietary protein. Aqueous RBPJ (density, 20%) was prepared by Kagome Co., Ltd. (Tokyo, Japan) (Table 2). The RBPJ was freeze-dried before being used in the experimental diet.

Table 1. Experimental diet composition.

| Experiment | I | II | III |
|------------|---|----|-----|
| Groups     | CAS20 | SPI50 | SPI35 | SPI20 | CAS20 | SPI20 | SPI20 |
| (g/kg diet) | | | | | | | |
| CAS        | 227 | 585 | 410 | 234 | 227 | 227 | 227 |
| SPI        | 403.5 | 45.5 | 220.5 | 396.5 | 403.5 | 326.9 | 323.9 | 403.5 |
| RBPJ       | 132 | 132 | 132 | 132 | 132 | 132 | 132 |
| Sucrose    | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Lard       | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Cellulose powder | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Mineral mixture | 35 | 35 | 35 | 35 | 35 | 35 | 35 |
| Vitamin mixture | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Choline bitartate | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Total      | 1,000 | 1,000 | 1,000 | 1,000 | 1,000 | 1,000 | 1,000 |

1 This diet was used for the sham operation group in all experiments. 2 CAS, casein. Crude protein contains 88.1 g/100 g, as is (Oriental Yeast Co.). 3 SPI, soy protein isolate (Fuji Oil Co.). 4 Crude protein contains 85.4 g/100 g, as is. 5 Crude protein contains 87.0 g/100 g, as is. 6 RBPJ, red bell pepper juice. 7 AIN-93G mineral mixture was used (Oriental Yeast Co.). 8 AIN-93 vitamin mixture was used (Oriental Yeast Co.).

Table 2. Ingredients in aqueous RBPJ.

| Ingredients | RBPJ1 |
|-------------|-------|
| Energy (kcal/100 g) | 74.0 |
| Moisture (g/100 g) | 80.0 |
| Carbohydrate (g/100 g) | 15.1 |
| Protein (g/100 g) | 2.20 |
| Ash (g/100 g) | 1.30 |
| Fat (g/100 g) | 0.40 |
| Dietary fiber (g/100 g) | 0.30 |
| Potassium (mg/100 g) | 699 |
| Sodium (mg/100 g) | 4.2 |
| Vitamin B6 (mg/100 g) | 0.91 |
| Capsanthin (mg/100 g) | 12.0 |
| Quercetin2 | 1.51 |

1 RBPJ, red bell pepper juice (RI=20%). 2 Quercetin concentration is presented as ppm/10 Brix aqueous solution.
were provided ad libitum throughout the duration of the experiments. The sham group was fed the CAS20 diet. Food intake and body weight were recorded twice per day. After 2 wk of feeding with the experimental diets, the sham group underwent sham operation, and the other groups underwent surgical right sciatic nerve resection. After surgery, food intake and body weight were recorded daily. Four days after surgery, the right anterior tibialis muscle was obtained from all mice groups under isoflurane anaesthesia. All samples were stored at −80°C until analysis.

Experiment I: dose-dependent effect of SPI on muscle atrophy: To evaluate the dose dependence of SPI, the amounts of SPI in the diet were set to 50%, 35%, or 20% (SPI50, SPI35, or SPI20 groups, respectively).

Experiment II: impact of a diet including RBPJ and SPI: We evaluated the influence of the RBPJ mixed diet (CAS20+RBPJ] group or SPI20+RBPJ group, respectively) on muscle atrophy markers.

Experiment III: effects of a diet including RBPJ and SPI on metabolites in denervated skeletal muscle tissue: We performed metabolome analysis to evaluate whether the diet including RBPJ and SPI affects metabolites in muscle tissue compared with CAS alone as the control. Samples (n=3) representing each group were selected based on muscle atrophy markers in the sham, CAS 20, and SPI 20 + RBPJ groups for metabolome analysis.

Quantitative real-time RT-PCR. Total RNA from each muscle sample was isolated using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) and the total RNA concentration was measured with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was prepared with PrimeScript™ RT Master Mix (TaKaRa Bio Inc., Shiga, Japan). Real-time RT-PCR was performed using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA) with an ABI PRISM 7300 sequence detection system instrument (Applied Biosystems) using the TaqMan method. Ready-to-use primers (TaqMan Gene Expression Assays; Applied Biosystems) were used to assess muscle atrophy. The following primers were used: MuRF1 (muscle RING-finger protein 1, Trim63 [tripartite motif-containing 63], Mm01185221_m1) and MAFbx/atrogin-1 (muscle atrophy F-box, Fbxo32 [F-box protein 32], Mm00499523_m1). These genes are markers of muscle atrophy associated with a specific E3 ubiquitin ligase (14). GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Mm99999915_g1) was used as a housekeeping gene. The mRNA expression levels of these genes were normalized to that of GAPDH and expressed as the relative change compared with the sham group.

Metabolome analysis. Metabolome measurements were carried out by Human Metabolome Technologies (Tsuruoka, Japan). Muscle metabolites were measured by capillary electrophoresis time-of-flight mass spectrometry (CE-TOF MS) as described previously (15–17). Approximately 30 mg of frozen tissue was plunged into 1,500 µL of 50% acetonitrile/Milli-Q water containing internal standards (Solution ID: 304–1002; Human Metabolome Technologies) at 0°C in order to inactivate enzymes. The tissue was homogenized three times at 1,500 rpm for 120 s each using a tissue homogenizer (Micro Smash MS100R; Tomy Digital Biology Co., Ltd., Tokyo, Japan) and then the homogenate was centrifuged at 2,300 × g and 4°C for 5 min. Subsequently, 800 µL of the upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter at 9,100 × g and 4°C for 120 min to remove proteins. The filtrate was centrifugally concentrated and resuspended in 50 µL of Milli-Q water for CE-MS analysis. Briefly, CE-TOF MS analysis was carried out using an Agilent CE capillary electrophoresis system (Agilent Technologies, Santa Clara, CA), and the systems were controlled with Agilent G2201AA ChemStation software (version B.03.01 for CE). In this analysis, water-soluble metabolites (900 substances) were measured. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed using Human Metabolome Technologies’ proprietary software PeakStat and SampleStat, respectively.

Statistical analysis. Data are expressed as the mean± standard error of the mean. Statistical analyses were performed using SPSS software (version 12.0J; SPSS, Inc., Chicago, IL). When analysis of variance showed a statistically significant difference, multiple comparison analysis was performed using a Dunnett’s test (p<0.05, the CAS20 group is treated as a control group).

RESULTS

MuRF1 and MAFbx/atrogin-1 expression are influenced by a diet including a dietary protein source and RBPJ (Experiment I & II)

In experiment I, food intake was significantly low in the SPI50 group compared with the control group, but

| Table 3. Growth parameters and tissue weights in Experiment I. |
|---------------------------------------------|---|---|---|---|
|                                      | Sham | CAS20 | SPI50 | SPI35 | SPI20 |
| Initial body weight (g)            | 22.8±0.3 | 22.9±0.3 | 22.8±0.2 | 22.9±0.2 | 22.9±0.2 |
| Final body weight (g)              | 25.3±0.5 | 24.4±0.5 | 23.0±0.3 | 23.7±0.3 | 24.1±0.2 |
| Food intake (g/d)                  | 3.09±0.08 | 3.06±0.10 | 2.48±0.06 | 2.80±0.04 | 2.91±0.06 |
| TA muscle (g)                      | 0.049±0.000 | 0.046±0.000 | 0.046±0.001 | 0.046±0.000 | 0.046±0.000 |
| TA muscle (wt% L)                  | 0.193±0.005 | 0.187±0.003 | 0.201±0.002 | 0.196±0.002 | 0.194±0.003 |

Data are expressed as the mean± standard error of the mean. Statistics is using a Dunnett’s test (CAS20 group is used as a control. *p<0.05). 1 Tibialis anterior muscle with sciatic nerve resection. 2 g/100 g body weight.
the relative weight of the tibialis anterior muscle was significantly high in the SPI50 group as compared with the CAS20 group (Table 3). The mRNA expression levels of MuRF1 and MAFbx/Atrogin-1 were determined (Fig. 1A, B). The levels of the gene expression markers were significantly higher in the CAS20 group than in the sham group. A diet containing 20% SPI did not alter the mRNA expression level compared with a 20% casein-including diet (CAS20 group). When SPI comprised half of the diet, the muscle atrophy markers were significantly lower as compared with the control group.

As compared to the control group, food intake, final body weight, and the relative weight of the tibialis anterior muscle in the other groups were not changed in Experiment II (Table 4). Figure 1C and D show the influence of diets containing RBPJ and the different protein sources. Compared with the casein diet, the diet including RBPJ and casein had no preventive effect on muscle atrophy. The levels of muscle atrophy markers were significantly lower in the group administered the diet including RBPJ and SPI20 than in the control groups (Fig. 1C, D).

**Table 4. Growth parameters and tissue weights in Experiment II.**

|                | Sham | CAS20 | CAS20 + RBPJ | SPI20 + RBPJ |
|----------------|------|-------|--------------|--------------|
| n              | 8    | 8     | 8            | 8            |
| Initial body weight (g) | 21.4 ± 0.3 | 21.4 ± 0.3 | 21.4 ± 0.3 | 21.4 ± 0.3 |
| Final body weight (g)    | 22.9 ± 0.5 | 23.0 ± 0.3 | 23.4 ± 0.4 | 22.9 ± 0.3 |
| Food intake (g/d)        | 3.13 ± 0.06 | 3.02 ± 0.08 | 3.10 ± 0.09 | 3.12 ± 0.06 |
| TA muscle (g)            | 0.042 ± 0.001 | 0.043 ± 0.000 | 0.043 ± 0.000 | 0.043 ± 0.000 |
| TA muscle (wt% 2)        | 0.185 ± 0.003 | 0.187 ± 0.002 | 0.183 ± 0.002 | 0.186 ± 0.004 |

Data are expressed as the mean ± standard error of the mean. Statistics is using a Dunnett’s test (CAS20 group is used as a control). 1 Tibialis anterior muscle with sciatic nerve resection. 2 g/100 g body weight.
Fig. 2. MuRF-1 mRNA expression and metabolome analysis in sham, casein, and SPI+RBPJ groups. A. MuRF-1 gene expression levels. The horizontal bars in each group represent the mean values. The characters (‘a,’ ‘b,’ ‘c,’ ‘d,’ ‘e,’ ‘f,’ ‘g,’ ‘h,’ and ‘j’) are indicated as individual mouse in experimental groups, respectively. Statistics is using a Dunnett’s test (CAS20 group is used as a control. *p<0.05). B. Principal component analysis (PCA). PC1 and PC2 are categorized according to dependency degrees of 44.27% and 11.89%, respectively. Muscle atrophy treatment was clustered by PC1. PC2 clustered the difference between the diets in the muscle atrophy induction group. C. Heat map and hierarchical cluster analysis. Blue (‘c,’ ‘a,’ ‘b’), green (‘h,’ ‘g,’ and ‘j’), and red (‘d,’ ‘e,’ and ‘f’) columns represent the sham, SPI+RBPJ, and CAS groups, respectively. The RBPJ + SPI group was located closer to the sham group. The relative comparison of water-soluble substances (900 substances) is indicated by the color change from green (−2.5) to red (+2.5). Twenty substances extracted from the heat map were highly correlated with the muscle atrophy marker (Murf-1 gene expression level).
A diet including RBPJ and SPI influences metabolites in muscle according to metabolome analysis (Experiment III)

Figure 2A shows the gene expression level of MuRF1 on RT-PCR. The level of the muscle atrophy marker was significantly higher in the CAS20 group than in the sham group. However, the group administered the diet including RBPJ and SPI20 had a significantly lower muscle atrophy level compared with the CAS20 group.

The PCA data obtained by metabolomics profiling by CE-TOF MS are shown in Fig. 2B. Sham treatment and muscle atrophy induction treatment were distinguished by principal component 1 (PC1, 44.27%). Principal component 2 (PC2, 11.89%) was classified by dietary differences under muscle atrophy treatment conditions. HCA divided the sham, CAS, and SPI+RBPJ groups into different clusters, as shown in Fig. 2C. Therefore, the correlations between the expression level of MuRF1 mRNA and metabolite levels were analyzed, with the data showing negative correlations for 5 metabolites and positive correlations for 15 (Fig. 2C). In particular, glutathione and S-adenosylmethionine (SAM) were in the group of metabolites showing a strong positive correlation with the MuRF1 mRNA expression level. Although the levels of branched chain amino acids and proline were not different among the groups, hydroxyproline was negatively correlated with muscle atrophy.

DISCUSSION

The findings of this study indicate that a diet including RBPJ and SPI suppress the markers of disuse muscle atrophy, a type of sarcopenia observed in patients with locomotive syndrome. In general, locomotive syndrome may be prevented by a mechanism that suppresses protein breakdown, and therefore muscle atrophy, by blocking the ubiquitination of insulin receptor substrate-1 (IRS1), a regulatory protein in the insulin signaling pathway. The active site of casitas B-lineage lymphoma-b (Cbl-b), which is a ubiquitin ligase, is competitively blocked by a specific oligopeptide known as Cblin. Administration of Cblin suppresses IRS1 ubiquitination, prevents muscle atrophy, and retains normal insulin signals for muscle maintenance (18, 19). Interestingly, glycine, which is contained in 40% of SPI, possesses a sequence similar to the inhibitory peptide sequence of Cblin, and a 20% glycine diet prevents muscle atrophy (7, 12). Indeed, we found that a 50% SPI diet (containing about 20% glycine) suppressed gene expressions related with muscle atrophy, but it is impractical to consume half the diet as SPI.

Even for a 20% soy protein diet (containing about 8% glycine), the effect of the diet on muscle atrophy varies significantly depending on the other concurrently consumed ingredients. In the field of exercise and sports science, many studies have investigated the physiological action of co-ingestion (20, 21). For example, Bell et al. (21) reported that consumption of a multi-ingredient nutritional supplement twice daily increased muscle strength and lean body mass in elderly individuals. In the present study, muscle atrophy was prevented in mice fed a mixture of RBPJ and soy protein, but not in those fed a mixture of RBPJ and casein. Despite the low glycine intake conditions, our results showed that a diet including RBPJ and SPI may prevent muscle atrophy. A limitation of this study is that it did not involve a group fed only SPI, and thus we cannot reach a firm conclusion about the role of RBPJ.

The effects of a diet including RBPJ and SPI on metabolites in the muscle atrophy state were investigated by metabolome analysis. PCA and HCA clearly divided the three groups into different clusters. In particular, the patterns of metabolites in muscles were similar in the two groups subjected to sciatic nerve resection, but the RBPJ and SPI group showed results more like the sham group than the CAS group.

Oxidative stress is generally considered to be one of the mechanisms underlying muscle atrophy. It is distinct from the ubiquitin-proteasome pathway, indicating that muscle atrophy can be suppressed by simply blocking oxidation. Some studies have indicated that the level of the antioxidant glutathione is decreased in muscles with atrophy because of the high turnover rate of glutathione due to an increase in reactive oxygen species (22, 23). However, in our study, glutathione was highly detected after sciatic nerve resection. The muscle atrophy may be at an early phase in this experiment, explaining the large amount of glutathione detected. Further studies are necessary to assess the roles of antioxidative effects related to RBPJ ingredients such as capsanthin and quercetin.

Metabolome analysis indicated that 20 metabolites/molecules were highly correlated with MuRF1 mRNA expression. Elevated levels of energy metabolism-related molecules (UTP, UDP, GTP, GDP, CTP, CMP, UDP-glucose, and UDP-GalNAc) indicate that excess energy accumulates as muscle atrophy progresses. The consumption of a RBPJ and SPI mixture may regulate energy metabolism to prevent accumulation.

A recent study by Obata et al. (24) showed increased levels of SAM in aged Drosophila melanogaster (fruit flies), suggesting an association between SAM and longevity. Activation of the metabolic pathway of SAM suppressed an age-related increase in SAM, extending the lives of the flies. In our study, the levels of SAM were high at the condition of muscle atrophy, but a diet including RBPJ and SPI consumption brought a low level of SAM, suggesting that a diet including RBPJ and SPI impacts the activation of energy consumption and extension of lifespan. Further studies are needed to clarify how concurrent intake of SPI and RBPJ affects SAM and its enzymes.

Our results showed that a mixed diet of RBPJ and SPI suppress the gene expressions related with muscle atrophy induced by sciatic nerve resection. SPI and RBPJ more effectively prevent muscle atrophy markers induction when consumed concurrently than when consumed separately, suggesting that they might complement each other’s physiological actions. Further investigation is needed to reveal the effects of RBPJ and SPI consumption on aging and oxidative stress, in addition to their effects on muscle atrophy.
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Author contributions
Study conception and design: NT, MF, and TF. Acquisition of data: NT, MF, TI, and HS. Analysis and interpretation of data: NT, MF, TI, YI, and HS. Drafting the manuscript or revising it critically for important intellectual content: NT, MF, MO, TN, and TF. Writing the paper: NT and MF. Approving the final version of manuscript: NT, MF, TI, YI, HS, MO, TN, and TF.

NT and MF contributed equally to this work.

Disclosure of state of COI
All authors have no conflicts of interest to declare for the present study.

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