Effect of Different Dietary Protein Composition on Skeletal Muscle Atrophy by Suspension Hypokinesia/Hypodynamia in Rats

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Summary Under microgravity conditions similar to those in space, it is known that various nutritional and physiological changes in the body are induced. Especially in the aspect of nutrition, muscle atrophy is a characteristic phenomenon accompanying weightlessness. This study was conducted to investigate the ameliorated effect of muscle atrophy caused by suspension hypokinesia by using the soy protein isolate (SPI) as the protein source in comparison with casein. Male Wistar strain rats (8 wk old) were divided into two groups, each suspended with a suspension harness, and fed on a 20% SPI diet or a 20% casein diet for 10 d. The body weights of the suspended rats fed casein or SPI decreased similarly. The weight of the gastrocnemius and soleus muscle were decreased by suspension hypokinesia; however, the degree of the decrease of the muscle weights, especially soleus muscles, of rats fed the SPI diet was smaller than that of rats fed the casein diet. Serum N2-methylhistidine concentration was significantly lower in rats fed the SPI diet than in rats fed the casein diet. Similarly, the activities of muscle protein-degrading enzymes such as calpain and proteasome were significantly lower in rats fed the SPI diet than in rats fed the casein diet. Cathepsin B+L activities were not affected by the SPI or the casein diet. Therefore it is suggested that SPI caused a reduction of the proteolysis of myofibrillar protein in skeletal muscles through a reduction of calpain and proteasome activities, in consequence to ameliorate the muscle atrophy.

Key Words muscle atrophy, soy protein isolate, N2-methylhistidine, calpain, proteasome

Muscle atrophy is a characteristic symptom of the prolonged hypokinesia resulting from restricted movement, prolonged bed rest, limited muscular function, and immobilization (1–6). On reduced load-bearing of the skeletal muscles under conditions of weightlessness, muscle atrophy has also been demonstrated (2, 3). It is very important to study the mechanism underlying the hypokinesia/hypodynamia effect on skeletal unloading because these examinations will contribute to the health of crews and their safety before, during, and after space missions. However, the mechanisms underlying muscle atrophy and the recovery involved and the beneficial countermeasures against it are not sufficiently understood.

The intracellular proteolytic process is composed of lysosomal and nonlysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpain, cysteine proteases in the cytosol, is thought to be the main agent of nonlysosomal Ca2+-dependent proteolysis that exists within the myofibril and which is capable of carrying out the initial step in myofibrillar proteolysis (7, 8). Proteasome, multicatalytic proteinase complexes in the cytosol that need ubiquitination of their substrates, is responsible for nonlysosomal ATP-dependent proteolysis (9). Although proteasome has been implicated in the regulation of myofibrillar protein degradation (10), its substrates and the control of its activity have not been fully studied in muscles. However, intact myofibrillar proteins are not substrates of proteasome (11), which may degrade muscle proteins released because of calpain action. Cathepsins, the main agents of lysosomal degradation, have been well established as contributors to muscle protein breakdown (12). Lysosomal proteases may degrade sarcoplasmic proteins and release myofibrillar proteins (13), but the precise roles of all these protein degradation systems in skeletal muscle are yet to be determined.

In this study, we measured the activities of muscle protein-degrading enzymes (calpain, proteasome, and cathepsins) and the blood concentration of N2-methylhistidine to investigate the ameliorative effect of muscle atrophy caused by suspension hypokinesia, using the SPI as the dietary protein source in comparison with casein.

MATERIALS AND METHODS

Animal experiments. Male Wistar strain rats at 8 wk of age were purchased from Japan SLC Inc. (Shizuoka, Japan). They were individually kept in wired cages in a temperature- and humidity-controlled room (24±1°C and 55±5% relative humidity) with a 12 h light-dark cycle (light on 07:00–19:00). The animals were di-
vided into two groups, one fed ad libitum a 20% casein diet and the other a 20% SPI diet (Table 1). After a 4-d adaptation period under light anesthesia with ethyl ether, every rat in the two groups was placed in a suspension harness made of denim. The denim suits were fastened to the backs of the caged animals with fastener tape and attached to a swivel suspended by chains. This suspension apparatus was a modification of the type used by Musacchia et al. (14). The rats could use their forelimbs to eat food and drink water; their hindlimbs hung free and were thus nonload bearing. The rats were lifted in metabolism cages and fed their respective diets for 10 d. On the last day in the experiment, the rats were killed by decapitation, and blood collected from the cervical wound and serum were stored at −20°C. The skeletal muscles (gastrocnemius and soleus) were quickly removed, frozen in liquid N₂, and stored at −80°C until protease analyses.

The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the University of Shizuoka.

**Chemical analysis.**

N,mid-methylhistidine analysis: Serum N,mid-methylhistidine concentration was analyzed by the method of Hayashi et al. (15) with a modification. The serum was applied to a cation-exchange resin column (7×60 mm, Dowex 50×8, 200 to 400 mesh, pyridine form) to separate most of the acidic and neutral amino acids from N,mid-methylhistidine with 20 mL of 0.2 m pyridine; N,mid-methylhistidine was eluted with 20 mL of 1 m pyridine. The eluent containing N,mid-methylhistidine fraction was then evaporated, and residue was dissolved in 1 mL of mobile phase (15 mm sodium octane sulfonate in 20 mm KH₂PO₄), and this was used for HPLC analysis. The HPLC analysis—by the use of a JASCO PU-1580 chromatography system equipped with a Shim-pack CLC-ODS column (6×150 mm) attached to an oven at 45°C. A JASCO model FP-920 fluorescence detector with excitation wavelength of 348 nm and emission wavelength of 460 nm was used to monitor the fluorescence product.

Measurement of protease activities: Gastrocnemius and soleus muscles were homogenized in 2 volume of ice-cold 20 mm Tris-HCl buffer (pH 7.5) containing 5 mm EDTA and 10 mm dithiothreitol. The homogenate was then centrifuged for 30 min at 30,000×g at 4°C. The supernatant was used for the measurement of calpain and proteasome activities. The resulting protein pellet was suspended in 50 mm acetate buffer (pH 5.0) containing 0.2 m NaCl and 0.1% Triton X-100. The suspension was centrifuged for 30 min at 13,000×g at 4°C. The supernatant was used for the measurement of cathepsin B+L activity. Calpain activity was measured by the method of Sasaki et al. (16) by the use of 0.2 mm succinyl-Leu-Leu-Val-Tyr-MCA, fluorogenic synthetic peptide as a substrate at pH 7.3. Proteasome activity was analyzed by the method of Tanaka et al. (17), determined with succinyl-Leu-Leu-Val-Tyr-MCA as a substrate at pH 8.0. The activity of cathepsin B+L was measured by the method of Barrett and Kirschke (18) by the use of fluorogenic peptides. Cathepsin B+L activity was assayed with 10 µm Z-Phe-Arg-MCA as a substrate at pH 5.5. This synthetic substrate is hydrolyzed by cathepsin B and cathepsin L, so the activity is shown as cathepsin B+L activity.

Proteins were measured by Lowry's method with bovine serum albumin as a standard (19).

**Statistical analysis.** Data were analyzed by Student’s t-test. A p value of <0.05 was considered to be statistically significant. Each result is expressed as the means±SE.

### RESULTS AND DISCUSSION

This model consists of a suspended rat whose weight was transferred to the abdominal surface, the forelimbs and hindlimbs being nonload-bearing for 10 d. In this condition, weightlessness and muscle atrophy are observed on hypokinesia (20,21). Recently, Ikemoto et al. reported that activate the ATP-ubiquitin-dependent proteolytic pathway and increase cathepsin L mRNA in the skeletal muscles of the tails of suspended rats (22). In our previous study (23), the fractional rate of protein synthesis decreased in the gastrocnemius muscle. but in the soleus muscle it did not change after 10 d of suspension. On the other hand, the rate of protein degradation in gastrocnemius and soleus muscles of suspended rats increased significantly. It is known that in a weightless condition, the skeletal unloading caused by space flight (24), bed rest (25), or hindlimb elevation (26) results in osteopenia (27). In the present experiment, the ameliorated effects of muscle atrophy were investigated in comparison with casein by the use of SPI as the dietary protein source.

Recently, SPI or its products have been widely used as protein sources. Sulfur amino acids are the first limiting amino acids and threonine is the second in both SPI and casein (28) because the amino acid compositions of SPI and casein resemble each other. But cystine, arginine, and glycine are included about twofold in SPI compared with casein. On the other hand, methionine and proline are included about half in SPI in comparison with casein though SPI and casein, 20% in diet, are

| Ingredient | 20% casein (%) | 20% SPI |
|------------|----------------|---------|
| Casein¹    | 20             | —       |
| Soy protein isolate² | —             | 20      |
| Corn starch³ | 41.90          | 41.90   |
| Sucrose¹   | 20.95          | 20.95   |
| Cellulose¹ | 5              | 5       |
| Corn oil   | 5              | 5       |
| AIN-76 vitamin mixture³ | 2          | 2       |
| AIN-76 mineral mixture³ | 5          | 5       |
| Choline-Cl | 0.15           | 0.15    |

¹ Supplied by Oriental Yeast, Tokyo, Japan.
² Supplied by Fuji Oil Co. Ltd., Osaka, Japan.
³ Supplied by Nihon Nosan Co. Ltd., Yokohama, Japan.
satisfied with the requirement of dietary amino acids.

The body weight and skeletal muscle weights of the suspended rats fed casein or SPI were presented in Table 2. In each group, the body weight was decreased by a similar suspension of hypokinesia. The gastrocnemius and soleus muscle weights were decreased by a 10-d suspension. However, the degree of muscle weight decrease, especially the soleus muscle, in rats fed an SPI diet was smaller than in rats fed a casein diet.

\[ N\textsuperscript{\text{-}}\text{methylhistidine} \]

is an amino acid produced by the posttranslational modification of histidine residues on actin and myosin molecules. There is no corresponding tRNA or oxidative pathway of this amino acid, making it a marker of degradation of actin and myosin (29). Generally, the urinary excretion of \( N\textsuperscript{\text{-}}\text{methylhistidine} \) is used as an index of skeletal muscle protein breakdown in the intact animal. Nagasawa et al. (30) have reported that plasma \( N\textsuperscript{\text{-}}\text{methylhistidine} \) is a sensitive index of myofibrillar protein degradation. Therefore we consider that the measurement of serum \( N\textsuperscript{\text{-}}\text{methylhistidine} \) concentration is a useful index of the status of myofibrillar protein breakdown in the present study. The serum concentration of \( N\textsuperscript{\text{-}}\text{methylhistidine} \) was significantly decreased by 33.1% in rats fed an SPI diet compared with a casein diet (Table 3). This result indicates that SPI reduces myofibrillar proteolysis in suspended rats.

The activities of muscle protein degrading enzymes were determined in gastrocnemius and soleus muscles (Table 3). Calpain activity was lower in the muscles of rats fed an SPI diet compared with a casein diet (Table 3). This result indicates that SPI reduces myofibrillar proteolysis in suspended rats.

Proteasome activity was also lower in the muscles of rats fed an SPI diet than in those of rats fed a casein diet; especially in the gastrocnemius muscle, it was significantly lower in the SPI diet than that in the casein diet. Calpain has been implicated in the degradation of the myofibrillar elements of muscle (7, 8). Calpain activation may trigger a dissociation of myofibrillar elements.

Proteasome activity was also lower in the muscles of rats fed an SPI diet than in those of rats fed a casein diet. Especially in the soleus muscle, it was significantly lower by about half in an SPI diet compared with a casein diet. Proteasome is a multicatalytic ATP-dependent proteolytic system (9). The multiple proteolytic pathways are essential to complete proteolysis.

The activities of cathepsin B+L were not significant in the muscles of rats fed an SPI diet compared with a casein diet. Lysosomal cathepsins B and L are endopeptidases thought to play major roles in intracellular proteolysis (31). Lysosomal proteases may degrade released myofibrillar proteins (14). An activation of the ubiquitin-proteasome proteolytic pathway has been found in many kinds of muscle-wasting conditions (32, 33). However, evidence for the activation of either lysosomal or Ca\( ^{2+} \)-dependent proteolysis is lacking in several kinds of muscle atrophy. The coordinated activation of three major proteolytic systems, namely, the lysosomal, Ca\( ^{2+} \)-dependent, and ATP-ubiquitin-dependent pathways, seems to be responsible for muscle atrophy.

Glucocorticoid is well-known as a stress hormone, in taking part in an oxidative stress. In general, stress induces the formation of free radicals and develops oxidative stress, initiating lipid peroxidation in tissues. Numerous studies have shown the relationships between glucocorticoid status, antioxidative defense systems, and lipid peroxidation. Slater et al. (34) reported an increase of intracellular peroxides and a decrease in glutathione when rat thymocytes were exposed by a methylprednisolone. Furthermore, dexamethasone has been reported to repress basal and stimulated manganese superoxide dismutase (Mn-SOD) mRNA and protein levels in IEC-6 cells (35). Lipid peroxidation is intimately involved in the pathogenesis of cellular injury by oxygen radicals (36). Therefore the free radicals may act the occurrence of muscular dystrophy (37). Skeletal muscle proteins could be damaged (38) or modified (39) by oxidative stress. We reported that the serum glucocorticoid concentration increased in rats that were exposed to suspension hypokinesia (40). Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic pathway by enhancing the expression of components of this system such as ubiquitin (41, 42). Oxidative stress has recently been suggested to act as a common mediator of several system of apoptosis (43). In our previous study, apoptosis was observed in the muscle cells of the suspended rats (unpublished data). Antioxidant \( N\text{-acetyl-}

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**Table 2. Effect of casein and SPI on body weight and skeletal muscles weights of rats after 10 d of suspension hypokinesia/hypodynamia.**

|          | Casein | SPI  |
|----------|--------|------|
| Body weight (g) | 189.1±7.9 | 196.1±5.1 |
| Gastrocnemius muscle weight (g) | 2.03±0.11 | 2.07±0.05 |
| Soleus muscle weight (g) | 0.084±0.004 | 0.103±0.008 |

Values represent means±SE, n=6. A Student’s t-test was performed. It was not significant in this level (p<0.05).

**Table 3. Effect of casein and SPI on blood concentration of \( N\textsuperscript{\text{-}}\text{methylhistidine} \) and skeletal muscle protease activities of rats after 10 d of suspension hypokinesia/hypodynamia.**

|          | Casein | SPI  |
|----------|--------|------|
| \( N\textsuperscript{\text{-}}\text{methylhistidine} \) (nmol/mL serum) | 22.1±1.9 | 14.8±1.9* |
| Calpain (nmol AMC/h/g protein) | | |
| Gastrocnemius muscle | 347.5±15.3 | 256.6±21.8* |
| Soleus muscle | 515.8±55.6 | 459.6±41.4 |
| Proteasome (nmol AMC/h/g protein) | | |
| Gastrocnemius muscle | 28.2±8.1 | 25.5±5.5 |
| Soleus muscle | 411.9±86.4 | 211.9±40.0* |
| Cathepsin B+L (nmol AMC/h/g protein) | | |
| Gastrocnemius muscle | 65.2±2.3 | 55.8±4.9 |
| Soleus muscle | 172.8±14.1 | 184.4±8.6 |

Values represent means±SE, n=6. A Student’s t-test was performed. *Significantly different from the casein diet (p<0.05).
cysteine strongly inhibited oxidative stress-induced apoptotic cell death (44). Generally, soybean includes an isoflavones, such as genistein and daidzein. Therefore SPI includes minor amounts of isoflavones. Soy isoflavones have been shown to exhibit antioxidant effects in vitro and in vivo by the direct free radical quenching ability (45, 46). Genistein and daidzein have also been known to prevent 8-hydroxy-2'-deoxyguanosine formation in cells and DNA exposed to oxidants (47). So SPI might reduce oxidative stress and apoptotic cell death in the muscle, though this was not determined in the present study.

In conclusion, the present results indicate that SPI caused a reduction of the proteolysis of myofibrillar protein in skeletal muscles through the reduction of calpain and proteasome activities, which will result in an amelioration of muscle atrophy.

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