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Increased oxidative properties of gastrocnemius in rats fed on a high-protein diet

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Abstract

It has been reported that a high-protein diet containing 30% protein partially prevents skeletal muscle fiber type changes (slow to fast) under atrophic conditions. No studies have examined the detailed effects of dietary protein on the oxidative properties of skeletal muscles. We examined the effects of a high-protein diet on the oxidative properties of rat gastrocnemius. Nineteen male Wistar rats (5 weeks old) were divided into the following groups: (1) control diet [15% protein (15P); $n=6$], (2) 25% protein (25P; $n=6$) and (3) 35% protein (35P, $n=7$). After 4 weeks of feeding, succinate dehydrogenase (SDH) staining and myosin isoforms were analyzed, along with the expression of the major transcription-related molecules for fast-to-slow fiber transition, i.e., peroxisome proliferator-activated receptor gamma coactivator (PGC) 1α and nuclear factor of activated T cells (NFAT) c1. SDH staining showed that the relative oxidative fiber content of the 35P group was significantly higher than that of the 15P group. The slow myosin heavy chain content in the 35P group was also higher than that in the 15P group. Western blotting analysis showed that the gastrocnemius of the 35P group contained significantly higher amounts of PGC1α and NFATc1 than that of the 15P group. We conclude that a high-protein diet, i.e., 35% protein, induces oxidative properties in rat gastrocnemius.

Keywords: Skeletal muscle; Peroxisome proliferator-activated receptor gamma coactivator 1α; Nuclear factor of activated T cells c1

1. Introduction

Proteins are one of the major nutritional elements in food. Young growing humans and animals require a high concentration of protein in their diets in comparison to adults. It has been noted that growing rats require approximately 15% dietary protein to meet their protein requirement and approximately 5% dietary protein for maintenance [1–5]. Following the recommendations of the Food and Agriculture Organization/World Health Organization/United Nations University international working group, the protein requirement for male and female adult humans was assumed to be 0.6 $g \cdot kg^{-1} \cdot d^{-1}$ of high-quality highly digestible protein [6]. Millward and Jackson [7] generated reference ratios of protein energy/total energy requirement (reference P/E ratio) and showed that it ranges from 0.126 for the UK omnivore diet to 0.054 for the rice-based diet of adults in West Bengal, demonstrating that 5–12% protein/total energy requirement meets the requirement demand for adult humans.

Information on the oxidative properties of skeletal muscles is useful to endurance runners for enhancing oxygen uptake. The number and activities of mitochondria play a key role in oxidative metabolism. The proliferation of mitochondria occurs in muscles in response to endurance exercise training [8] and thyroid hormones [9], while the loss of mitochondria is associated with inactivity [10] and aging [11]. These metabolic changes usually accompany fiber type change. In microgravity environments such as space flights, fiber type distribution changes from slow (oxidative) to fast (glycolytic). Mitochondrion proliferation and fast-to-slow fiber transition usually occur as adaptations to endurance exercise. It has been also reported that aging induces a change in the myosin heavy chain (MHC) from fast to slow with a decrease in the cross-sectional area in Type II fibers [12].

Nutritional control of the muscle fiber type has also been reported. The restriction of dietary intake (normal, 840 kJ/kg body weight per day; restricted, 280 kJ/kg body weight per

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day) reportedly affects pig myosin isoform compositions during early postnatal development (from 6 to 9 weeks of age) [13]. Muscles rich in fast-twitch myosin isoforms lose greater mass and protein content during intake deprivation than those in which slow-twitch isoforms predominate. Brodsky et al. [14] showed that a 4-week isoenergetic dietary protein deprivation (normal, 1.67 g/kg fat-free mass/day; low protein, 0.71 g/kg fat-free mass/day) induces a fast-to-slow transition in adult human MHC isoform proportions. These findings suggest that low energy intake and/or dietary protein depletion influence the MHC.

On the other hand, a high-protein diet is considered to either promote muscle hypertrophy or prevent muscle atrophy because proteins and/or amino acids (particularly leucine) reportedly stimulate the mammalian target of rapamycin and up-regulate protein synthesis in skeletal muscles [15,16]. Taillandier et al. [3] attempted to prevent muscle atrophy in the soleus muscles of rats (average body weight, 150 g) subjected to 21 days of hindlimb unloading by administering a high-protein diet (30%). Although this could not prevent muscle atrophy, they observed that a high-protein intake partially preserved fiber type distribution, i.e., prevented conversion from slow- to fast-twitch fibers in rat soleus muscles. They confirmed by pathological examination that the unloaded soleus in the group fed on a diet of 30% protein contained significantly more, i.e., 70.7% more Type I fibers than that in the hindlimb-unloaded rats fed on a diet of 15% protein (50.2% of Type I). Their observation suggested that a moderately high protein diet (30% protein) affects the fiber type composition of skeletal muscles, but detailed examination has not been pursued previously under normal conditions. Based on the observations of Taillandier et al., we can speculate that a high-protein diet may make muscle fibers more oxidative.

Metabolic adaptations largely result from a coordinated hormonal and genetic response. The oxidative adaptation of skeletal muscles involves the physiological processes of biogenesis and increase in the number of mitochondria, increased glucose uptake, fiber type transition, etc. Some important regulators have been reported to exist. The peroxisome proliferator-activated receptor gamma coactivator (PGC) family of proteins has been identified as the central family of transcriptional coactivators involved in the induction of mitochondrial biogenesis [17]. In particular, PGC1α has been reported to promote mitochondrial biogenesis and fiber type transformation in skeletal muscle cells [18,19]. Besides the coactivators, several transcriptional elements involved in fast-to-slow transition have been also reported. In particular, nuclear factor of activated T cells (NFAT) proteins are directly bound by calcineurin and dephosphorylated; thus, their nuclear translocation and activation is promoted [20]. The NFAT family contains NFATc1–c4. In particular, NFATc1 (NFAT2) is reported to play a key role in myosin isoform change [21]. Calcineurin-mediated NFATc1 activation has been reported to be involved in the up-regulation of slow MHC gene expression during the fast-to-slow transformation of skeletal muscle cells [22–24].

In the present study, we examined the effects of a 4-week high-protein diet on the metabolic properties and MHC isoforms in rat gastrocnemius muscles. As described above, we hypothesized that a high-protein diet promotes oxidative characteristics in skeletal muscles based on the results of Taillandier et al. [3]. We employed two parameters to examine the metabolic change in muscles, namely, (1) succinate dehydrogenase (SDH) activities for analyzing metabolic characteristics and (2) the electrophoretic separation of MHC isoforms. We also evaluated the expression of both PGC1α and NFATc1 to examine whether a high-protein diet affects the expression of two major transcriptional coactivator molecules that modulate the oxidative capacities and myosin isoform compositions.

2. Methods and materials

The protocols used in this experiment were approved by the ethical committee of Nippon Sports Science University.

2.1. Animals and diets

Nineteen male Wistar rats (4 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed individually in a 12-h light/dark cycle with the lights kept on from 7:00 PM to 7:00 AM. Water was given ad libitum during the experiments.

After 1 week, the rats were assigned to three groups. After another 4 weeks (the final age was 9 weeks), each group was fed the following: (1) the control diet containing 15% protein and 71.3% carbohydrate (61% cornstarch and 10% sucrose) (15P; $n=6$), (2) the diet containing 25% protein and 41.3% carbohydrate (31.3% cornstarch and 10% sucrose) (25P; $n=6$) and (3) the diet containing 35% protein and 31.3% carbohydrate (21.3% cornstarch and 10% sucrose) (35P; $n=7$) (Table 1). The 15P control diet was equivalent to the American Institute for Nutrition (AIN)-93M diet, which was developed as an appropriate diet for rodents by the AIN [5], and the other diets were modified from the control diet. The food was administered in a dry pellet form. The protein was

Table 1
Design of diets

Ingredients	Diet (%)		
	15P (control)	25P	35P
Casein	15	25	35
Corn starch	61	51	41
Sucrose	10	10	10
Soybean oil	4	4	4
Cellulose	5	5	5
Mineral mixture	3.5	3.5	3.5
Vitamin mixture	1	1	1
L-Cystine	0.18	0.18	0.18
Choline (bitartrate)	0.25	0.25	0.25
t-Butylhydroquinone	0.0008	0.0008	0.0008

supplied as casein, carbohydrate as cornstarch or sucrose and fat as soybean oil. The AIN-93 vitamin and AIN-93 mineral mixtures were obtained from Oriental Yeast (Tokyo, Japan), as shown in Table 1.

The weight of each animal was recorded once a week throughout the experimental period. After the experimental period, the animals were fasted for 5 h, anesthetized with isoflurane and exsanguinated. The target tissues and organs were removed immediately after death. After measuring the mass of each organ, all specimens, including serum, were rapidly frozen in liquid N_2 and stored at −80°C until use.

2.2. SDH staining

The quickly frozen muscle serial cryosections (10 μm thick) were examined for SDH activity, which is an indicator of the mitochondrial oxidative potential, as reported by Hirofuji et al. [25]. The SDH activity was determined in an incubation medium containing 100 mM phosphate buffer (pH 7.6), 1.5 mM nitroblue tetrazolium and 48 mM succinate disodium salt. The reaction was arrested by multiple washings in distilled water, and the sections were dehydrated in a graded series of ethanols, passed through xylene.

The SDH activities of muscle fibers from the deep (close to the bone) and superficial (close to the surface of the muscle) regions of the muscle were examined. These muscle regions were selected for analysis because the gastrocnemius muscle shows an increasing gradient of fibers that have a high oxidative enzyme activity proceeding from the superficial to the medial deep region of the muscle. The tissue sections were digitized as gray-level images on a computer-assisted image-processing system (Scion Image, Scion). Each pixel on the computer was quantified as 1 of 256 gray levels. A gray level value of zero was equivalent to 100% transmission of light (%T), and that of 255 was equivalent to 0%T. The optical density (OD) value was determined based on the gray levels. We classified all the muscle fibers into three groups, namely, slow oxidative (SO) (%T: 100%–80%), fast oxidative glycolytic (FOG) (%T: about 60%–40%) and fast glycolytic (FG) (%T: 10%–0%) based on the OD values. We examined the tissue sections of all the animals (15P: $n=6$, 25P: $n=6$, 35P: $n=7$) in a similar manner. A total of 100 fibers were analyzed per region.

2.3. Electrophoretic separation of MHC isoforms

The electrophoretic separation of MHC isoforms was essentially the same as that performed by Talmadge and Roy [26]. The 4% stacking gels and 8% separating gels contained 30% glycerol. The running conditions were 2 mA (constant current) at 4°C for 24 h. The gels were stained with Coomassie blue. The stained gels were photographed and analyzed using a densitometer (CS analyzer, ATTO).

2.4. Western blotting analysis

Frozen muscle specimens were homogenized for 30 s in a mixture of 50 mM Tris-Cl (pH 7.4), 5 mM EDTA, 10 mg/ml PMSF, 0.5 mg/ml leupeptin, 0.2 mg/ml aprotinin, 0.1% Triton X-100, 0.2% Nonidet P-40, 0.05% mercaptoethanol and 1 mM Na_3VO_4. The homogenate was centrifuged at 15000*g* for 25 min at 4°C. The supernatant was removed, and its protein concentration was determined using a protein concentration determination kit (Protein Assay II, Bio-Rad). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (stacking gel: 3%; separating gel: 7.5%; protein: 30 μg per lane) was performed under reducing conditions. After the electrophoresis, the separated proteins were electrically blotted onto a polyvinylidene difluoride membrane.

After blotting, the membrane was washed in phosphate-buffered saline containing 0.1% Tween 20 (tPBS). After blocking with 5% skimmed milk (Difco Skim Milk, BD Pharmingen), the membrane was incubated with the primary antibodies at 4°C overnight. After washing with tPBS, the membrane was reacted with peroxidase-conjugated secondary antibodies (Pierce) at room temperature for 1 h. Chemiluminescent reagents (Supersignal West Dura; Pierce) were used for the detection. All antibodies used in this study were purchased (PGC1α: AB3242, Chemicon; NFATc1: MA3-024, Affinity Bioreagent).

An image was obtained using a chemiluminescence detector (AE6961, ATTO), and the signal obtained was quantified using the same chemiluminescence detector and PC application (CS analyzer, ATTO).

2.5. Statistical analysis

All the results are expressed as the mean±S.D. One-way analysis of variance (ANOVA) and Bonferroni's post hoc test were used to determine the significance of difference. We used the 2-tailed Student's *t* test to compare two groups. Significance was accepted at $P<.05$.

3. Results

3.1. Food consumption, body mass and tissue weight

Food consumption is shown in Table 2. In the 4-week experimental period, the food consumption of the 15P group in the first week was significantly higher than that of the other two groups. No significant difference was observed in the total energy intake throughout the experimental period among the three groups.

The body mass, tissue weight and fat content are listed in Table 3. Only the body mass after the feeding was significantly different between the 15P and 35P groups.

Table 2

	15P	25P	35P
Pretreatment (g/day)	17.2±0.7	16.7±1.1	17.3±0.6
1 week (g/day)	18.0±1.2	16.1±0.4*	16.6±0.8**
2 weeks (g/day)	18.7±0.8	17.5±1.2	17.6±1.4
3 weeks (g/day)	21.0±0.6	20.0±0.8	20.4±1.0
4 weeks (g/day)	19.9±1.4	20.5±1.0	18.8±1.0
Total (g/day)	19.6±0.6	18.8±0.5	18.6±1.0

*$P<.05$; **$P<.01$ against 15P, one-way ANOVA and Bonferroni's post hoc test.

Table 3
Body weight and tissue masses

Body mass (g)	Pre treatment	15P	25P	35P
		168.2±6.8	163.6±2.3	161.4±11.4
	Post treatment	340.6±10.3	329.2±7.3	320.4±13.2*
Fat tissues				
Mesenterium (g)		5.2±1.2	5.2±0.7	4.9±0.5
Hypogastrium (g)		5.3±0.7	4.9±1.0	4.7±0.7
Retroperitoneal (g)		9.7±1.4	9.6±1.6	9.1±1.3
Gastrocnemius (mg)		1616.4±65.6	1548.3±92.1	1535.9±122.7
Soleus (mg)		141.0±5.9	138.1±4.0	140.9±1.3

*P<.05 against 15P.

Skeletal muscle masses are also shown in Table 3. There was no significant difference between the groups.

During the experimental period, there were no apparent diet-dependent differences in activities and behaviors, suggesting that there was little or no difference in the energy expenditure among the groups.

3.2. SDH staining analysis

Table 4 shows the quantitative examination of the oxidative fibers. The deep region of the medial gastrocnemius (MG) contained more oxidative fibers than the superficial region of the lateral gastrocnemius (LG). With regard to the deep region of the MG, the relative content of FOG fibers in the 35P group was significantly higher than that in the 15P group. With regard to the superficial region of the LG, the 35P group had a significantly lower number of FG fibers than the 15P group. There were no significant differences in the soleus muscles.

We confirmed that significant differences could only be observed between the 15P and 35P groups. Thereafter, further analysis was focused on the differences between these groups.

3.3. Myosin heavy chain composition of gastrocnemius muscles

The electrophoretic separation and quantitative analysis of MHC isoforms are shown in Fig. 1. and Table 5. The Type I chain content of the 35P group was significantly higher than that of the 15P group.

Table 4
Distribution of oxidative fibers in the gastrocnemius and soleus muscles

		15P	25P	35P
Deep MG (%)	FG	24.5±11.3	12.3±19.5	2.3±6.0
	FOG	37.2±12.8	43.2±13.6	50.9±9.8*
	SO	38.3±6.3	44.5±15.2	46.9±6.4
Superficial LG (%)	FG	43.7±5.5	38.0±8.6	29.9±8.7*
	FOG	30.3±9.6	29.8±7.7	35.6±11.3
	SO	26.0±7.9	34.1±7.5	35.1±5.8
Soleus (%)	FG	0	0	0
	FOG	78.6±5.7	68.1±6.5	72.8±5.8
	SO	21.3±3.9	31.9±6.5	27.2±5.8

*P<.05, one-way ANOVA and Bonferroni's post hoc test.

3.4. Western blotting analysis of PGC1α

Fig. 2A shows the representative western blot detection of PGC1α in the gastrocnemius muscles. The densitometric analysis is shown in Fig. 2B. The PGC1α level was significantly increased in the gastrocnemius muscles in the 35P group compared to those in the 15P group.

3.5. Western blotting analysis of NFATc1

Fig. 3A shows the representative western blot detection of NFATc1 in the gastrocnemius muscles. Multiple bands near 132–78 kDa were confirmed. Based on the report by Gooch et al. [27], we designated the higher molecular weight band as phosphorylated NFATc1 and the lower band as dephosphorylated NFATc1. The densitometric analysis is shown in Figs. 3B and C. Regardless of the molecular weight, the 35P group contained a significantly greater amount of NFATc1 than the 15P group.

4. Discussion

A high-protein diet significantly induced SDH activities in the gastrocnemius (Table 4). On the other hand, there was no significant difference in the soleus. Taken together, the regions that were originally rich in oxidative fiber tended not to be affected by a high-protein diet. Ishihara et al. [28] examined the SDH activities in the deep, medial and superficial regions of the tibialis anterior muscles in mice after 4 weeks of voluntary running exercise. They found that the mean SDH activities in the medial and superficial regions

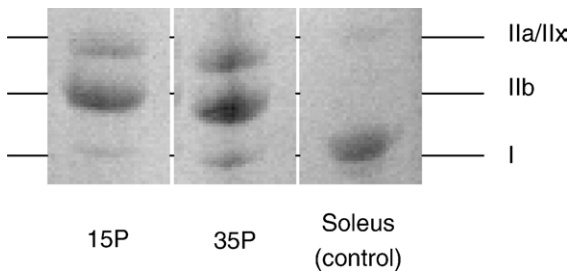

Fig. 1. Electrophoretic separation of myosin isoform. The representative result of the electrophoretic separation of the myosin isoforms is shown. The soleus muscles are used as the control for Type I myosin chains.

Table 5
Compositions of myosin isoforms

	15P (n=6)	35P (n=6)
MHC I (%)	5.7±1.7	10.7±2.9*
MHC IIa/IIx (%)	35.1±4.9	35.0±4.0
MHC IIb (%)	59.1±5.5	54.2±5.7

*P<.05 against 15P, Student's t test.
MHC, myosin heavy chain.

were greater in the exercise group than those in the control group. There was no significant difference in the deep region. We observed that the SDH activities of the mixed gastrocnemius muscles in the 35P group were greater than those in the 15P group. This adaptation is fairly similar to that observed in exercised muscles, suggesting that oxidative adaptation under a high-protein diet similar to that induced by exercise. Adaptation depending on the muscle group is also similar to that observed by Ishihara et al.

In this study, a significant increase in the percentage of MHC I (slow) (from 5.7%±1.7% to 10.7%±2.9%) under the 35P diet was also confirmed. It is well recognized that exercise training and chronic electrical stimulation induce skeletal muscle fiber type and MHC isoform transformation [IIB (fast glycolytic)→IIX (fast intermediate)→IIA (fast oxidative)→I (slow)] [29]. In a short duration of high-intensity endurance exercise training and chronic low frequency electrical stimulation, only the fiber type transformation within the subtypes of Type II fibers was observed. In a long duration of exercise, the alternation from Type II to Type I may possibly occur [30]. A diet containing 35% dietary protein induces an MHC isoform change from MHC II to MHC I in rat gastrocnemius muscles, as observed in the long duration of exercise. In addition, a high-protein diet may induce oxidative properties as well as MHC isoform change in rat skeletal muscles.

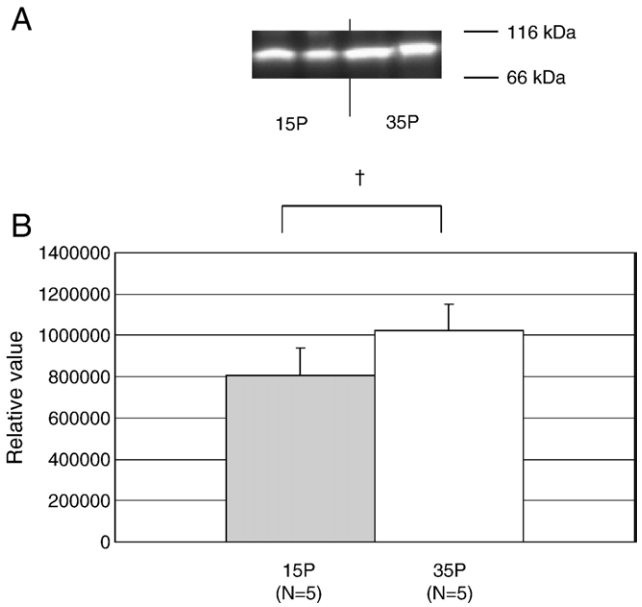

Fig. 2. Western blot analysis of PGC1α in the gastrocnemius muscles. The representative blots obtained using an anti-PGC1α antibody (A). The average values of PGC1α in the gastrocnemius muscles (B). All the results are expressed as the mean±S.D. †P<.05.

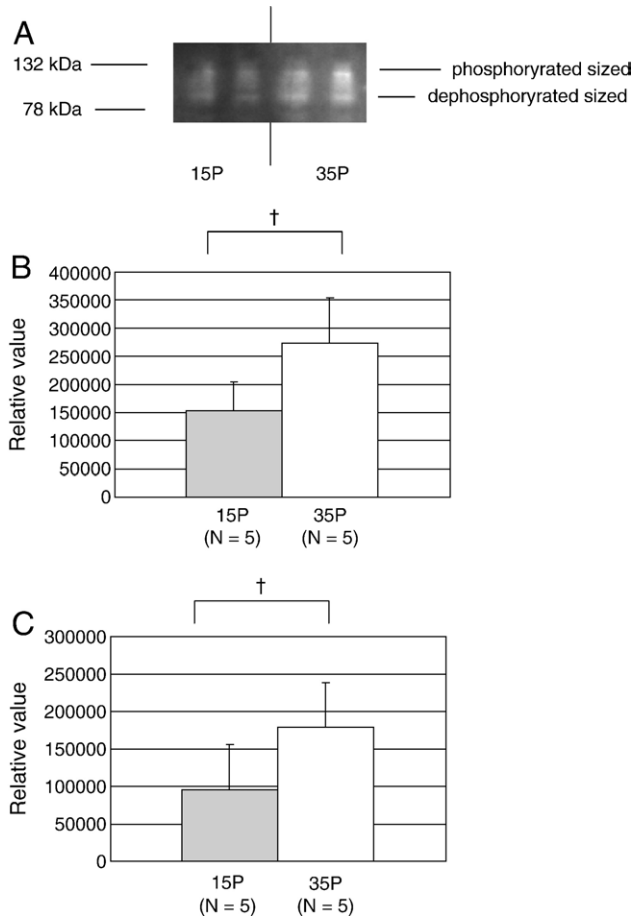

Fig. 3. Western blot analysis of NFATc1 in the gastrocnemius muscles. The representative blots obtained by using an anti-NFATc1 antibody (A). The average values of phosphorylated-sized NFATc1 in the gastrocnemius muscles (B). The average values of dephosphorylated-sized NFATc1 in the gastrocnemius muscles (C). All the results are expressed as the mean±S.D. (P<.05).

In order to further characterize the effects of a high-protein diet, we examined the expression of PGC1α, which controls mitochondrial biogenesis and oxidative enzyme activities [17,19]. As shown in the results, the PGC1α protein content in the rat gastrocnemius muscles of the 35P group was significantly higher than that of the 15P group. Overexpression of PGC1α induces an increase in the mitochondrial respiratory chain-related mRNA expression of nuclear respiratory factor (NRF)-2α and COX II as well as COX IV [18]. PGC1α overexpression is also reported to induce a shift in the muscle fiber type from fast to slow [19]. Taken together, the increased oxidative properties of skeletal muscles under a high-protein diet accompany increased PGC1α expression. Increased PGC1α should facilitate the induction of oxidative properties in skeletal muscles of the animals fed on a high-protein diet.

We confirmed that the content of NFATc1 increased significantly under a high-protein diet. Swoap et al. [24]

demonstrated that the overexpression of NFATc1 in C2C12 myotubes did not activate muscle-specific promoters. Liu et al. [31] also demonstrated that exogenous NFATc1 GFP-fusion protein was localized at the sarcomeric Z lines in adult mouse myofibers. Electrical stimulation caused the phosphorylation of NFATc1 and, as a result, localization in the nuclei. These reports suggest that not the increase in the NFATc1 content but phosphorylation contributes to the fast-to-slow fiber type transformation. On the other hand, we also confirmed that the content of phosphorylated-sized NFATc1 proteins in the 35P group was significantly higher than that in the 15P group. We are now primarily considering that the 35P diet increases the NFATc1 content (including phosphorylated-sized one), and as a result, a shift from fast myosin to slow occurs.

White et al. and Brodsky et al. [13,14] showed that undernutrition and low protein intake induce fast-to-slow transition in the MHC. On the other hand, we found evidence showing that a high-protein diet induces fast-to-slow muscle fiber type conversion. Why both high-protein and low-protein diets induce fast-to-slow fiber type transition remains unknown. Brodsky et al. discussed that the conversion of skeletal muscle from a more energy-costly to a less energy-costly isoform composition during energy deprivation is intuitive during protein deprivation [14]. We also consider that similar mechanisms underlie the age-related fast-to-slow change in the MHC [12]. On the other hand, it has been reported that the protein fraction of the diet is one of the main determinants of diet-induced thermogenesis [32,33] and plays a key role in body weight regulation through satiety related to diet-induced thermogenesis [33]. Thus, we consider that a high-protein diet increases thermogenesis and leads to high oxidative properties. Although further examination is necessary, we hypothesize that low- and high-protein diets have similar results with different mechanisms.

We also noted that the significantly lower body mass of animals in the 35P group compared with the 15P group was confirmed in this study. Although we did not observe any significant differences, the average adipose tissue and skeletal muscle masses of the 35P group were lower than those of the 15P group. We have observed the same tendency in our latest study on the elevated synthesis of myostatin, which strongly prevents muscle hypertrophy, under a high-protein diet [4]. We consider that statistical significance will be observed when a larger sample size is increased. In addition, the protein fraction of the diet is one of the main determinants of diet-induced thermogenesis, as depicted above [32,33]. We primarily hypothesize that a high-protein diet increases energy expenditure, decreases fat tissues and induces oxidative properties in skeletal muscles.

Aging and physical activity contribute to inducing oxidative properties into skeletal muscle [12]. In this study, all the animals were housed individually in an isolated plastic case (25 cm width ×40 cm length ×20 cm height), and they were not subjected to exercise intervention. In addition, the ages of all the animals matched in this study. Primarily, nutritional difference was the only controlled valuable in this study. Although we should take into account many factors that may influence the results, we are now considering that nutritional interference induces metabolic differences in skeletal muscle tissues.

In conclusion, a high-protein diet induces oxidative properties in rat skeletal muscles as well as a fast-to-slow MHC isoform shift. These phenomena are also accompanied with the elevated expression of both the translation coactivator PGC1α and the transcriptional factor NFATc1.

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