

⟨Original Article⟩

Effect of exercise on muscle protein and mitochondrial function in mice model of skeletal muscle atrophy

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Summary The purpose of this study was to produce a mouse model of muscle atrophy by hindlimb suspension and examine the effect of exercise periods on muscle protein and mitochondrial functions. The study subjects were 20 wild-type mice, which were randomly divided into four groups according to difference in exercise periods after hindlimb suspension. As a result, the gene expression of Fis1 of mitochondria showed significantly high values ($p < 0.001$), and latent anti-oxidant potential showed significantly low values ($p < 0.01$) in the group that exercised five times/week for one week. Moreover, the gene expression of S100-b showed significantly low values ($p < 0.05$) in the group that exercised five times/week for three weeks. These results suggest that different exercise periods influence the factors that induce inflammatory response, fission of mitochondria, and latent anti-oxidant potential.

Key words: Exercise, Muscle proteins, Mitochondrial, Skeletal muscle atrophy

1. Introduction

Aging and loss of activity, muscle atrophy due to undernutrition, and skeletal muscle metabolic disorder have all been identified as causes of skeletal muscle abnormality. Skeletal muscle abnormality is classified into two types; 1) imbalance of muscle protein composition and decomposition and 2) mitochondrial dysfunction. Muscle atrophy is a

phenomenon caused by the activation of decomposition resulting in a decrease in muscle protein in cells¹. Three protein pathways, the ubiquitin proteasome pathway, autophagy pathway, and calpain pathway, are factors that play important roles in the decomposition of muscle protein and are involved in muscle protein decomposition. Some ubiquitin transferases that are expressed in muscle specificity have been reported for muscle atrophy². MuRF1 (muscle RING finger protein-1) and Muscle Atrophy

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F-box/Atrogin-1 (following MAFbx) are important markers (hereinafter called the muscle atrophy-related gene). Previous studies have reported that the expression of the muscle atrophy-related gene increases during muscle atrophy, and that muscle atrophy is reduced in knockout mouse³. Moreover, MAFbx and TNF- α (tumor necrosis factor) decrease with exercise after hindlimb suspension, and muscle atrophy is improved.

Generally, oxidative stress is defined as a balance between ROS (reactive oxygen species) and anti-oxidant materials. It has been reported that H₂O₂, which is a ROS, functions as a signal transmitter in cells and is related to MuRF1⁴. However, functions of ubiquitin transferase interact with other ubiquitin transferases, and muscle atrophy occurs in association with autophagy pathway or oxidative stress. Therefore, in order to understand exercise effects on muscle atrophy, their relationship between mitochondrial function and oxidative stress needs to be discussed, as well as the decomposition system of muscle protein. Skeletal muscle is full of mitochondria and the dynamics such as fission and fusion are repeated to maintain its functions. Also, low-quality mitochondria are removed by autophagy. The balance between fission and fusion maintains the form of mitochondria. A network is formed by the activation of fusion and fragmented mitochondria are formed by activation of fission⁵. Moreover, it has been pointed out that mitochondria multiply through fission, and fission is particularly important for association of local mitochondria to neurites⁶. Further, such “mitochondria fission-appropriate mitochondria arrangement in cells” has not been clarified for skeletal muscles. Generally, persistent exercise increases the number of mitochondria and causes changes in their form⁷. Moreover, it has been reported that exercise raises the expression of fusion and increases muscle protein, while no changes are recognized in fission⁸. However, although exercise has an influence on increase in the number of mitochondria, it has been reported from different studies that changes are recognized or not recognized in fission⁹. Therefore, questions remain regarding the molecular mechanism. Furthermore, the influence of exercise

periods on the mitochondrial functions has not been clarified for muscle atrophy.

In this study, considering the mitochondrial functions; fission protein 1 (Fis1), dynamin related protein 1 (Drp1), mitofusin 2 (Mfn2), and optic atrophy 1 (Opa1) play important roles for fission and fusion, respectively. Inference of morphological changes of mitochondria is estimated by measuring these four types of muscle protein. Therefore, in this study, the authors aimed to create a muscle atrophy model for hindlimb suspension and clarify the influence of exercise periods on muscle protein and mitochondrial functions.

2. Material and Methods

2.1. Animals

The subjects in this study were 20 wild-type mice (C57BL/6NCrSlc, male, 10 weeks old) that were randomly divided into four groups (n = 5 for each) shown below. Further, they were divided by the presence of exercise after hindlimb suspension (two weeks of suspension) and randomly divided into four groups by combining the 1- and 3-week periods). The categories were: Group A: hindlimb suspension with exercise (exercise 5 times/week for 1 week), Group B: hindlimb suspension without exercise (1 week), Group C: hindlimb suspension-with exercise (exercise 5 times/week for 3 weeks), and Group D: hindlimb suspension without exercise (3 weeks).

2.2. Protocol

All mice were bred for generalization for two weeks from the age of 10 weeks, and hindlimb suspension was performed from the age of 12 weeks for 2 weeks (until the age of 14 weeks). Exercise was initiated after the completion of hindlimb suspension, with its frequency, duration, and intensity based on past studies (Frequency: 5 times/week, Duration: Start at 15 min/day with gradual increase of 3 min/day up to 60 min/day, Intensity: 18-19 m/min, 5% slope)¹⁰. Mice in Groups A and B were sacrificed at 15 weeks, and those in Groups C and D were sacrificed at 17 weeks. Moreover, mice were

sacrificed 5 h after the completion of exercise, based on procedures in past studies. The lateral head of gastrocnemius was collected and RNA was stabilized (RNAlater Stabilization Solution, Thermo Fisher Scientific, Japan) and stored at -20°C until analysis. Blood sampling (approximately 100 μL) was performed and the sampled blood was promptly centrifuged to collect blood plasma, which was then refrigerated before the d-ROMs test. All mice were kept in an environment with light and dark cycles of 12 hours (7-19:00), at a room temperature of $20^{\circ}\text{C}\pm 1$, and could freely access food pellets with no restriction on their behavior.

2.3. Real-time polymerase chain reaction (PCR)

In the mRNA analysis, the total RNA was extracted using the phenol-chloroform extraction method according to the manufacturer's instructions (RNeasy Lipid Tissue Mini Kit, Qiagen, Germany). The cDNA composition was performed by High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Japan). For real-time PCR, Taqman Probe method was applied for 40 cycles with a PCR analysis system (Chromo4, Bio-Rad, USA). Fifteen factors were assumed for target genes, and GAPDH was used as an endogenous standard gene (Table 1). For the Ct (cycle threshold) values, their relative value was calculated by the Comparative Ct method ($\Delta\Delta\text{Ct}$ method) and compared with $\Delta\Delta\text{Ct}$ of Group B as a reference value (1.0).

2.4. Grip strength and body weight

For muscular strength, maximum values of limbs were continuously measured five times (10 s rest between measurements) with small animal grip measuring equipment (GPM-100B, Melquest Ltd., Toyama, Japan), and average values were calculated. Measurements were performed before hindlimb suspension (Pre) and at the time of sacrifice (Post). The mice were weighed with an animal scale (Type KN, Natsume Seisakusho Co., Ltd).

2.5. Oxidative stress regulation system

Using analytical equipment for reactive oxygen and free radicals (FRAS4/FREE, H & D, Italy), we

Table 1 Primer sequences used for quantitative real-time PCR

Mouse Sequence(ABI TaqMan Gene expression assays)	
Gene name	TaqMan probe set ID
MuRF-1	Mm01185221_m1
MAFbx/atrogen-1	Mm00499523_m1
AMPK	Mm01264789_m1
PGC-1 α	Mm00447183_m1
ERR- α	Mm00433143_m1
NRF-1	Mm01135606_m1
NRF-2	Mm00477784_m1
TFAM	Mm00447485_m1
S100-b	Mm00485897_m1
Opa1	Mm01349707_g1
Mfn2	Mm00500120_m1
Fis1	Mm00481580_m1
Drp1	Mm01342903_m1
GAPDH	Mm99999915_g1

MuRF1; muscle RING finger protein-1, MAFbx; Muscle Atrophy F-box/Atrogen-1, AMPK; AMP-activated protein kinase, PGC-1 α ; Peroxisome Proliferator-activated receptor- γ coactivator-1 α , ERR- α ; estrogen receptor related factor a, NRF-1; nuclear respiratory factor 1, NRF-2; nuclear respiratory factor 2, TFAM; transcriptional factor A mitochondrial, S100-b; S-100B protein, Opa1; optic atrophy 1, Mfn2; mitofusin 2, Fis1; fission protein 1, Drp1; dynamin related protein 1, GAPDH; housekeeping gene.

measured oxidative stress with the reactive oxygen metabolites test (d-ROM test) and anti-oxidant potential with the biological anti-oxidant potential (BAP) test. The latent anti-oxidant potential (BAP/d-ROM ratio) was calculated. In the d-ROM test, the levels of free radicals in the body, especially hydroperoxide concentrations, were measured (carratelli units (U.CARR), 1 U.CARR = 0.08 mg/dL of hydrogen peroxide) according to the optical measurement method (color reaction), and the measured value indicated the degree of oxidative stress (oxidative reaction)^{10,11}. Meanwhile, in the BAP test, the levels were measured in micrometers ($\mu\text{M mol/L}$) by the reduction action of anti-oxidant materials in blood plasma, and the measured values indicated the degree of anti-oxidant potential (anti-oxidant reaction). Thus, the amount of blood plasma that was reduced to ferrous ions (when mixed with reagents containing ferric ions) was measured at the decoloring level of the color reaction liquid according to the optical measurement method. The

content of iron ions to which blood plasma was reduced was the anti-oxidant potential.

The BAP/d-ROM ratio, which was calculated based on the values obtained in the BAP test and the d-ROM test, indicated the degree of latent anti-oxidant potential. Thus, the latent anti-oxidant potential indicated the balance between oxidative stress and anti-oxidant potential^{10,11}.

2.6. Statistical analysis

SPSS (Ver21.0 for win) was used for statistical analysis. Significance tests were performed by ANOVA, multiple comparison, Wilcoxon signed-rank test, and Spearman rank correlation coefficient. The study was conducted upon the approval of the research promotion committee for experiments on animals of Saitama Prefectural University (NO.27-11).

3. Results

For MuRF-1 and MAFbx, gene expression of Group D tended to be higher (1.7 times and 1.8 times, respectively, the same applies hereafter) than

that of Group C (0.9 times and 1.2 times, respectively).

Moreover, PGC-1 α tended to be higher in Group C (2.5 times) than Group D (1.3 times). S100-b was significantly lower in Group C (0.1 times) than Groups D (0.5 times) and B (1.0 time) ($p < 0.05$ and $p < 0.001$, respectively), while no significant difference was seen for NRF-2 (Figure 1). For Fis1 of mitochondria, Group A (6.9 times) showed significantly higher values than Group B (1.0 time) ($p < 0.001$), while no significant difference was seen for Opa1 (Figure. 2). For Drp1, there was a tendency for similarity to Opa1. These results showed that gene expression of Fis1 was high in Group A, and S100-b was low in Group C. Moreover, it was shown that the muscle atrophy-related gene was low and PGC-1 α was high in Group C.

For muscular strength, comparison of Pre and Post demonstrated that muscular strength tended to greatly decrease in Group D, while values within the group were dispersed. Moreover, a relationship between muscular strength and the muscle atrophy-related gene was not seen. No significant difference was found in weight among the four groups (Table

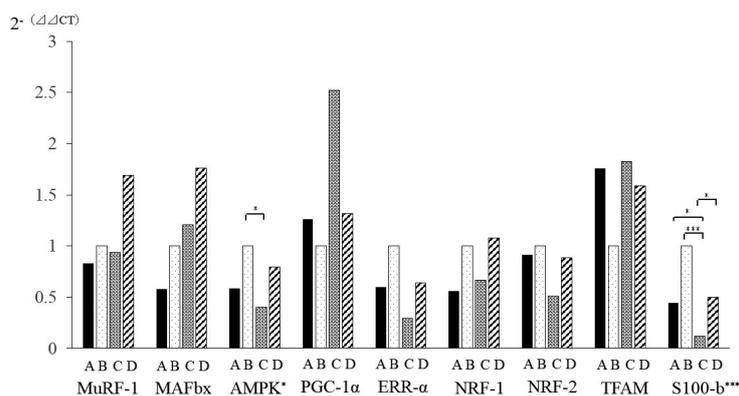


Figure 1 Effects of hindlimb suspension on expression levels of mitochondrial biogenesis-related genes. mRNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. * or *** indicate significant differences at levels of $p < 0.05$, $p < 0.001$, Group A: hindlimb suspension with exercise (exercise 5 times/week for 1 week), Group B: hindlimb suspension without exercise (1 week), Group C: hindlimb suspension- with exercise (exercise 5 times/week for 3 weeks), and Group D: hindlimb suspension without exercise (3 weeks).

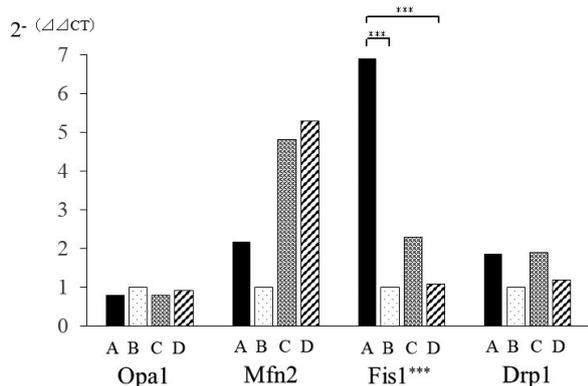


Figure 2 Effects of hindlimb suspension on mRNA expression levels of mitochondrial enzymes. mRNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. *** indicate significant differences at levels of $p < 0.001$, Group A: hindlimb suspension with exercise (exercise 5 times/week for 1 week), Group B: hindlimb suspension without exercise (1 week), Group C: hindlimb suspension- with exercise (exercise 5 times/week for 3 weeks), and Group D: hindlimb suspension without exercise (3 weeks).

2). For BAP/d-ROM ratio, Group A showed significantly lower values than Group B ($p < 0.01$), while there was no significant difference for d-ROM test (Table 2).

4. Discussion

Muscle mass of skeletal muscle is based on a balance between composition and decomposition. Muscle atrophy occurs when this balance is changed.

The causes of muscle atrophy are wide-ranging; yet, the expression of the muscle atrophy-related gene increases in most types of muscle atrophy, being involved in muscle protein decomposition¹. In temporal changes by hindlimb suspension, muscle protein decreased 5 h after hindlimb suspension, and decomposition of muscle protein was increased by the second week¹². Moreover, it has been reported that muscle protein decreased by 40% with hindlimb suspension of two weeks¹³. Since the expression of the muscle atrophy-related gene was high (1.7 times, 1.8 times) in Group D in this study, the possibility that decomposition of muscle protein was increased with hindlimb suspension, like in precedent studies¹², has been suggested. Exercise suppresses the expression of the muscle atrophy-related gene and controls the muscle protein decomposition system¹⁴. Moreover, it has been reported that electrical stimulation induces a temporal decrease in MAFbx for muscle atrophy by denervation¹³. Since expression of the muscle atrophy-related gene showed low values (0.9 times, 1.2 times) in Group C, it was suggested that the muscle protein decomposition system by exercise was possibly suppressed. Thus, tendencies like those shown in past studies are seen, because expression of the muscle atrophy-related gene is influenced by hindlimb suspension¹²⁻¹⁴.

Expression of PGC-1 α (peroxisome proliferator-activated receptor- γ coactivator-1 α) induced by exercise was related to the muscle atrophy-related gene, and expression decreased by hindlimb suspension¹⁵. It has been reported that PGC-1 α exerted an influence on mitochondrial composition and

Table 2 Change in the grip strength test, the oxidative stress regulation system and body weight

Group	Grip Strength Test (mg)		d-ROMs test (U.CARR)	BAP test ($\mu\text{M mol/L}$)	BAP/d-ROM ratio**	Body weight (g)	
	Pre	Post				Pre	Post
A	242.1 \pm 40.2	204.4 \pm 24.6	92.2 \pm 3.1	2472.4 \pm 177.7	26.82 \pm 1.75 ^{††}	24.8 \pm 1.1	25.6 \pm 1.4
B	219.5 \pm 46.8	229.1 \pm 17.4	83.0 \pm 7.2	2736.8 \pm 169.2	33.14 \pm 3.31	24.9 \pm 0.9	26.4 \pm 0.8
C	214.4 \pm 35.3	195.4 \pm 14.4	93.3 \pm 7.0	2604.8 \pm 144.5	28.05 \pm 2.50	25.5 \pm 1.1	27.2 \pm 0.4
D	210.2 \pm 21.4	168.1 \pm 39.0	89.5 \pm 4.7	2791.0 \pm 175.9	31.24 \pm 2.39	24.4 \pm 1.1	26.9 \pm 0.7

A Group: Exercise(1 week) after hindlimb suspension, B Group: 1 week(no exercise) after hindlimb suspension, C Group: Exercise(3 weeks) after hindlimb suspension, D Group: 3 week(no exercise) after hindlimb suspension, d-ROMs test: plasma oxidative stresses, BAP test: plasma anti-oxidant potential, d-ROM/BAP ratio : ratio of d-ROM to BAP.

Values are mean \pm SD. p Value by ANOVA test for the comparison between 4 groups and Bonferroni test for the d-ROMs test vs BAP test or d-ROM/BAP ratio, Body weight. Wilcoxon signed-rank test for the comparison between Grip Strength Test .

** $p < 0.01$, ^{††} $p < 0.01$, A Group vs B Group.

muscular fiber switching to slow muscle, promoting an increase in energy consumption and stamina¹⁶. In this study, PGC-1 α of Group C tended to be high (2.5 times), while the muscle atrophy-related gene showed low values. These results implied the possibility that expression of the muscle atrophy-related gene was suppressed by PGC-1 α expression, like precedent studies. Since the muscular strength was dispersed, its relationship with the muscle atrophy-related gene was not observed. In the muscular strength measurement method in previous studies, measurements were continuously performed six times, while the mouse was slowly pulled with one-min rests during the measurement cycle, and an average of the measured values was obtained¹⁷. Therefore, regarding the muscular strength measurement method, the need to conduct the study based on the precedent study¹⁷ has been presented.

Generally, the S100-B protein specifically expresses in astrocyte and assumes a role for survival of neuronal cells¹⁸. The possibility that an increase in the S100-B protein reflected continuous cell injuries and secondary cell injuries has been noted and is regarded as a factor for inducing an inflammatory response (alarmin)¹⁹. It has been reported that apoptosis was caused when the concentration of S100-B protein was increased and nitric monoxide and proinflammatory cytokine were released from glial cells, being involved with oxidative stress²⁰. In the current study, gene expression of S100-B was low in Group C (0.1 times), while significantly high values were shown in Groups D (0.5 times) and B (1.0 times). However, for d-ROM test and BAP/d-ROM ratio, no significant difference was seen between Groups C and D. S100-B was expected to have a relationship with oxidative stress, although it did not. The authors presume that d-ROM test is an analysis with plasma, and the gene expression of S100-B was analyzed with skeletal muscle; therefore, different samples were used for the analyses. Fluctuation of S100-B gene expression was within 1.0 time, or no change was recognized in Fis1 of mitochondria. In any case, the gene expression of S100-B was suppressed in Group C has been shown.

Mitochondria are dynamic cell organs that are bound by the fusion process and divided by the fission process. Increases in gene expression of Fis1 and activation of Drp1 have been reported as influences of exercise on mitochondria⁹. Moreover, it has been reported that in hindlimb suspension, the amount of muscle protein involved in fusion decreases, and the amount of muscle protein involved in fission increases²¹. It has also been reported that the amount of muscle protein involved in the fusion of mitochondria and gene expression of muscle protein of Mfn2 are increased by electrical stimulation and persistent exercise⁸. It has been reported from different studies that changes were recognized or not recognized in fission⁹. In the current study, significantly high values were seen for Fis1 in Group A, while a similar tendency was observed for Drp1. Thus, it was clarified that Group A was involved in fission of mitochondria, like the precedent studies. Moreover, the possibility that Group A was involved in the reorganization of a network of mitochondria by physical inactivity or in decomposition of damaged mitochondria, and influenced “mitochondria fission - appropriate mitochondria arrangement in cells” became clear. Further, it has been reported that Fis1 was a small molecule protein that existed with penetrating mitochondria adventitia and controlled fission of mitochondria as independent from Drp1²². It has also been reported that mitochondria produced more ROS when it was fragmented by fission²³.

In the current study, no difference was seen in d-ROM test between Groups A and B while BAP/d-ROM ratio was significantly lower in Group A. Thus, oxidative stress was high and anti-oxidant reaction was low in Group A, because BAP/d-ROM ratio indicated equilibrium between oxidative stress and anti-oxidant reaction. This caused a reduction of latent anti-oxidant potential. Therefore, one of the possibilities is that mitochondria became smaller pieces in Group A and eventually influenced oxidative stress and antioxidative potency, which caused a reduction of latent anti-oxidant potential.

Conflicts of Interest

The authors declare no conflicts of interest associated with this manuscript.

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