（論文題目）

Effects of an intermittent low-frequency electrical stimulation on skeletal muscle

（和　訳）

間欠的低周波数電気刺激が骨格筋に与える影響

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Chapter 1

General introduction

1.1 The condition to lead skeletal muscle hypertrophy

Skeletal muscle assumes physical movement and has highly plasticity. Skeletal muscle contraction under various physiological conditions induces morphological changes. Skeletal muscle hypertrophy is well-known adaptive response to muscle contraction and external stimuli. This response has multiple beneficial aspects from point of view of health science such as preventing injury, improving lipid (1) and glucose metabolism (2, 3). Thus, it is thought that skeletal muscle hypertrophy is important for improving quality of life in humans. For these reasons, it has been focused on importance of skeletal muscle hypertrophy in this thesis.

Resistance training is most helpful method for skeletal muscle hypertrophy. Generally, resistance training to lead skeletal muscle hypertrophy recommends at the intensity of over 70% of 1 repetition maximum (1RM) (4). In numerous studies, it has been reported that over 70 % 1RM training is often used to lead skeletal muscle hypertrophy (5). This is raised that muscle contraction with high force generation is important for skeletal muscle hypertrophy. On the other hand, recent studies suggest that low force with varied interposition such as blood flow restriction (6, 7), slow movement (8, 9), and with high repetitive contractions (10, 11) also induce skeletal muscle hypertrophy. Thus, the condition of leading to skeletal muscle hypertrophy is still under development.

1.2 Skeletal muscle hypertrophy in animal experimental models

In animal experiment, synergist ablation is often used to lead skeletal muscle hypertrophy. This experimental model is invasive i.e. synergist muscle surgically detached and subsequently induce compensatory hypertrophy in other synergistic muscles (12). Weight lifting model is also used for mimicking human resistance training (13, 14). In this model, animals were trained muscle against progressively heavier loads and, in return received a food reward. On the other hand, neuromuscular electrical stimulation (NMES) model also mimic resistance training (15, 16). Basically, NMES is one of the physical therapy methods and widely used for improving muscle function and strength training (15). NMES can represent various physiological conditions by changing their electronic frequencies. Electrical stimulus can be generated muscle force dependently their frequency (17-19).

Over 40 Hz electrical frequencies induce tetanic contraction (15) (Figure 1-1A). This frequency stimulation is intermittently imposed on skeletal muscle and consequently can induce subsequent hypertrophic adaptation. Several studies have shown that electrical stimulation with high frequency pulse succeeded in leading skeletal muscle hypertrophy (20-25). In human study, Gondin et al. (16) have indicated that isometric contraction with 75 Hz stimulation is provoked increasing maximal voluntary contraction (MVC), hypertrophic response and fast-to-slow transition in muscle fibers (summarized in Figure 1-1A). Their subsequent experiment has also suggested increasing MVC, however, despite fiber type transition, NMES cannot get oxygen capacities (26). Thus, it might be

thought that high frequency stimulation induced muscle hypertrophy is unrelated oxidative function.

In contrast, 10-20 Hz frequencies generate a twitch contraction (19, 27) (Figure 1-1B). NMES with low frequency pulse combined long term stimulation mimics endurance-like training and prominently expresses getting oxidative function related molecules such as Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), which are thought master regulator of mitochondria biogenesis and energy metabolism (28, 29) and phenotypic change such as fast to slow shift in muscle fiber type (27, 30, 31) (summarized in Figure 1-1B). Although endurance or –like exercise can get oxidative capacities, hypertrophic response is difficult to occur (32, 33).

Surprisingly, we found that low frequency electrical stimulation induced skeletal muscle hypertrophy depending on its stimulating condition (20). This model is completely different from commonly accepting NMES protocol because the exerted muscular force with low frequency NMES induces weaker than that with high frequency NMES. I think that this phenomenon is quite unique and, thus, I focused on this novel model for skeletal muscle hypertrophy in this thesis.

1.3 The molecular mechanisms in skeletal muscle hypertrophy

Skeletal muscle hypertrophy is occurred by increasing muscle protein synthesis (MPS) and its iteration (34) (Figure 1-2). MPS is regulated by intracellular signaling (35). Especially, it is thought that mammalian target of rapamycin complex 1 (mTORC1) is a pivotal regulator of increasing MPS

(36-38). Moreover, mTOR and its downstream target such as p70 K-Da ribosomal protein kinase (p70S6K), which is also well-known as indicator for skeletal muscle hypertrophy.

Phosphorylated p70S6K is correlated strongly with muscle mass after contraction induced hypertrophy (39). However, MPS related pathway is more than one signaling pathway. Mitogen activated protein kinase (MAPKs) pathway is also known to increase MPS (40-42). Notably, one of the MAPK, named Extracellular signal Regulated Kinase 1/2 (ERK1/2), can also phosphorylate other MPS related molecules. Moreover, ERK 1/2 and p70S6K have common molecule as downstream target, termed ribosomal S6 protein (rpS6). The phosphorylation of rpS6 is also known as MPS associated molecule (43) (Above signaling pathway is summarized in Figure 1-3). Recent study has shown that mTOR signaling is activated as contraction force is increased (44). Therefore, mTOR signaling activity is important for high intensity resistance training intended to skeletal muscle hypertrophy. Contrastively, recent study has suggested that p70S6K can phosphorylate by mTOR-independent manner (45). Although the phosphorylation of p70S6K can be indicated MPS, its upstream molecule may be erratic according muscle contractile condition. Taken together, MPS related signaling play an important role for skeletal muscle hypertrophy while detail of molecular mechanisms of skeletal muscle hypertrophy is still unclear.

1.4 The purpose of this thesis

Based on these backgrounds, the purpose of this thesis is decided as follows.

I) To develop skeletal muscle hypertrophy model by using NMES with low frequency electrical stimulation.

I tried to develop novel skeletal muscle hypertrophy model in animal by using low frequency electrical stimulation.

II) To investigate molecular mechanism for skeletal muscle hypertrophy.

I examined several MPS-related molecules expressions in acute bout of exercise with low-frequency NMES.

III) To explore the possibility that low-frequency electrical stimulation induces skeletal muscle hypertrophy in the subject failed to gain muscle hypertrophy with high-frequency electrical stimulation.

Skeletal muscle hypertrophy is not absolute response. It is thought that genetic background and individual difference also affect this response. Thus, I tried to induce skeletal muscle hypertrophy by using animals having the different genetic background.

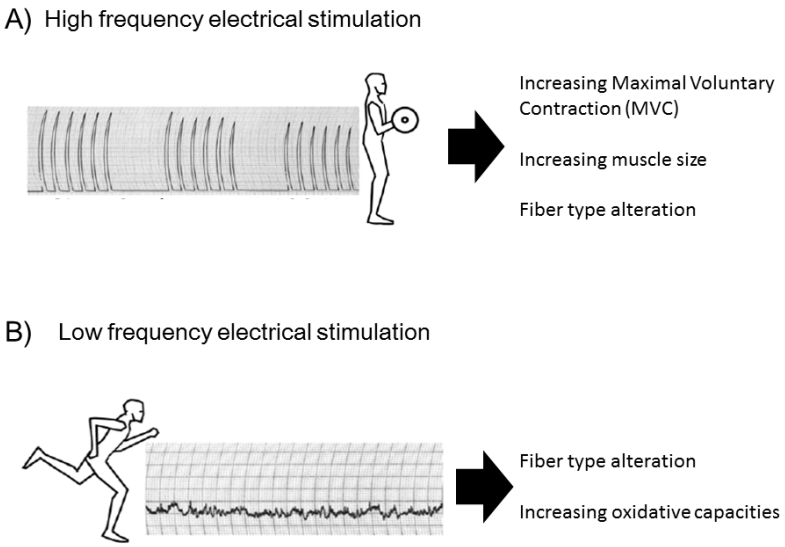

Figure 1-1.

(A) Typical effects by high frequency electrical stimulation (B) Typical effects by low frequency electrical stimulation (Illustration in this figure were taken from (27))

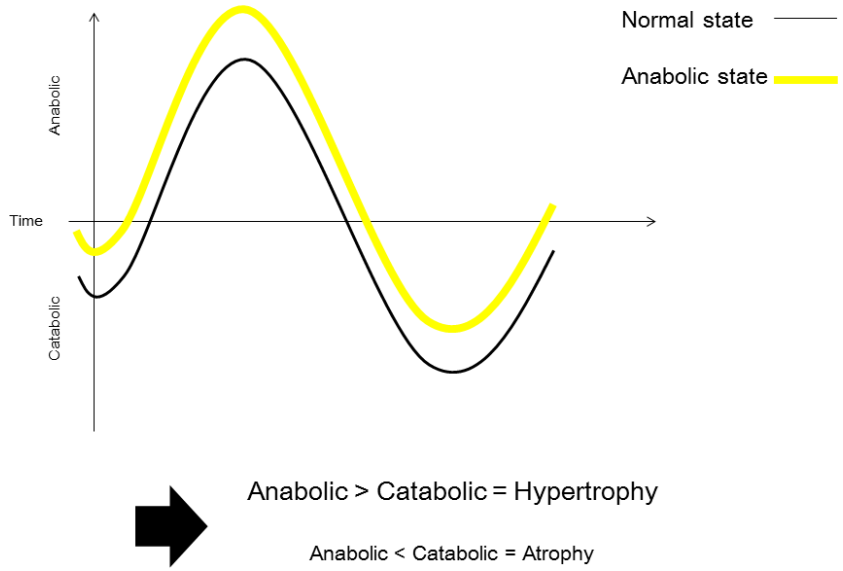

Figure 1-2.

The conceptual diagram of muscle protein synthesis. The black line represents normal state. The yellow line represents anabolic state after exercise. (Illustration was recasting from (46))

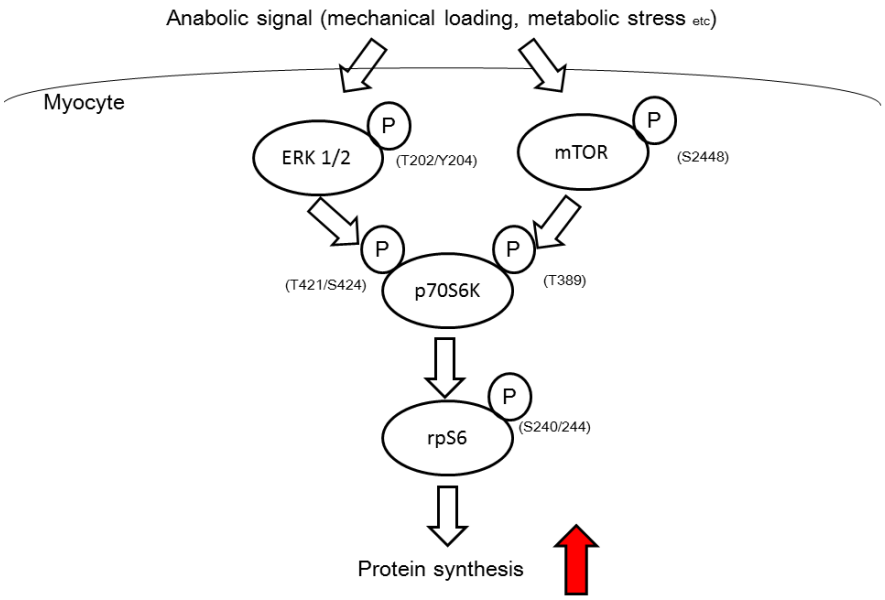

Figure 1-3. The brief pathway of protein synthesis

Illustration represents brief pathway of protein synthesis. mTOR: mammalian target of rapamycin. ERK 1/2: extracellular signal-regulated kinase 1/2. p70S6K: 70-KDa ribosomal protein kinase. rpS6: ribosomal protein S6.

Chapter 2

Effect of intermittent low-frequency electrical stimulation on the rat gastrocnemius muscle

INTRODUCTION

Skeletal muscle demonstrates high plasticity, responding to external stimuli with morphological changes. Skeletal muscle hypertrophy is a well-known adaptative response to external stimuli, such as resistance training. For humans, the American College of Sports Medicine recommends resistance training of over 70% of 1 repetition maximum (RM) to lead skeletal muscle hypertrophy (4). In muscle contraction leading to hypertrophy, type II fibers respond to external stimuli with a greater amount of hypertrophy than do type I fibers (47-49). Since this process is governed by the size principle, training to induce skeletal muscle hypertrophy demands high force in order to generate type II fibers in voluntary contraction. Conversely, several recent studies have reported that low-intensity (～30% 1 RM) training, in conjunction with high volume, also causes protein synthesis and skeletal muscle hypertrophy (10, 11). The most convincing hypothesis is that the later phases of exhaustive training regimens also recruit type II fibers, regardless of the training intensity. Taken together, the mobilization of type II fibers can be viewed as being critical for the induction of skeletal muscle hypertrophy.

Muscle hypertrophy is caused by a shift of the protein metabolic balance toward an anabolic state. This mechanism is regulated by intracellular signaling. Recent studies have shown that the mammalian target of the rapamycin complex (mTORC) signaling pathway is a key regulator of the protein synthesis rate and muscle size (36, 38). Furthermore, the state of phosphorylation of the

70-kDa ribosomal protein S6 kinase (p70S6K), one of the downstream targets of mTORC signaling, is frequently used as an indicator of training induced hypertrophy (39, 50, 51). The mitogen-activated protein kinase (MAPK) cascade has also been reported to be another anabolic pathway that is activated by mechanical stress, such as muscle contraction modes, and metabolic states (40, 52, 53). Specifically, the activation of extracellular-signal regulated kinase (ERK) 1/2, but not p38 MAPK, is crucial for the induction of muscle hypertrophy (40). Taken together, the mTORC1 and ERK 1/2 signaling molecules can be regarded as indicators of training-induced muscle hypertrophy.

Electrical stimulation of the skeletal muscle, typically termed neuromuscular electrical stimulation (NMES), is commonly used clinically to enhance the rehabilitation of skeletal muscle function. The force frequency relationship of NMES indicates that increases in the stimulation frequency result in increased muscle force production (54). In animal experimental muscle models for skeletal muscle hypertrophy, NMES is generally performed with intermittent and high frequencies (more than 60 Hz) (21, 55-57). On the other hand, when NMES was applied with a lower electrical frequency (～20 Hz) and long activation times, it led to higher oxidative properties and a fast to slow conversion of the muscle fiber phenotype (58). Therefore, animal experimental models with NMES were used to mimic resistance training and/or endurance exercise in order to test the effects of changes in frequency and stimulation time on muscle hypertrophy (27). Because of its

lower exerted force, low-frequency NMES has not been used as a stimulator for muscle hypertrophy. Since NMES randomly activates type I and type II fibers, independent of frequency (19), there might be a possibility that low-frequency NMES will lead to muscle hypertrophy in the same way that high-frequency does.

In the present study, I aimed to investigate whether intermittent activation with high (100 Hz) or low (10 Hz) frequencies of NMES could induce skeletal muscle hypertrophy in the rat. For this purpose, I conducted 2 experiments. First, I investigated whether acute bouts of exercise induced by low or high electrical frequencies of NMES could sway skeletal muscles toward anabolic states. I conducted histological and western blot analysis, focusing on the intracellular signaling phosphorylations of p70S6K (Thr389) and ERK 1/2 (Thr202/Tyr204). In a second experiment, I subjected rats to 6 sessions of training with low or high electrical frequencies, to investigate whether chronic NMES with different frequencies induces muscle hypertrophy.

Materials and Methods

Animal care

Thirty-six male Sprague-Dawley rats (9 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All animals were housed individually, in a 12-h light-dark cycle, with the lights kept on from 6:00 PM to 6:00 AM, and they were given food and water ad libitum. All procedures used in this study were approved by the ethical committee of the Nippon Sports Science University.

Experimental Design

Rats were divided evenly into a high-frequency electrical stimulation (HFS; 100 Hz) group and a low-frequency electrical stimulation (LFS; 10 Hz) group. They were then further randomly assigned to the following groups: (1) dissection immediately after training (Post0 group), (2) dissection 3 h after training (Post3 group), and (3) dissection after 6 sessions of training (6 sessions group). Thus, there were 6 groups defined by the frequency of electrical stimulation and the time points at which data were collected (n=6 each group).

Resistance Training Protocol

Under anesthesia, the right lower leg of each rat was shaved. The rats were then subjected to isometric training by electrical stimulation. Each rat was laid prone on a platform and its right knee

was extended with a dynamometer, with the ankle joint positioned at an angle of 90°. The triceps of the right leg muscle was then stimulated (voltage: 30-35 V; pulse duration 4 ms: frequency: 100 Hz for HFS group and 10 Hz for LFS group) with a surface electrode (7.5 mm × 7.5 mm) that was connected to an electrical stimulator and isolator (Nihon Koden, Japan). The left medial gastrocnemius muscle served as the untrained (UT) control.

For all training sessions, the triceps surae muscle was trained by stimulation for five 3s contractions, with a 5s interval between each contraction. Four sets in total were performed, with 3 min intervals between each set. All training sessions were conducted on every other day. The training and exercise schedule details are presented in Figure 1.

Animals were sacrificed immediately or at 3 or 24 h (6 sessions) after training. The medial gastrocnemius muscle was removed, weighed, and rapidly frozen in liquid nitrogen, and the right and left medial gastrocnemius muscles were triturated in liquid nitrogen and stored at – 80℃ until use.

Periodic Acid-Schiff (PAS) Staining and Immunohistochemistry

To investigate the intramuscular glycogen, I used a commercially available PAS staining kit (Muto Chemical Co., Ltd. Japan) and performed the protocol was according to the manufacturer's instruction manual.

To classify the fiber type, 10-μm-thick muscle cryosections were fixed with 2%

paraformaldehyde and 0.25% picric acid in 0.1 M phosphate-buffered saline (PBS), for 15 min at room temperature. Following fixation, the sections were washed in 0.1 M PBS for 15 min and then postfixed in ice-cold methanol (-20℃) for 10 min. The sections were then washed 3 times in 0.1 M PBS for 5 min, and blocked in a 0.1 M PBS solution containing 5% goat serum and 1% Triton-X-100, for 1 h at room temperature. After blocking, the primary antibodies were applied (Laminin and Fast myosin heavy chain; Sigma, USA) over night at 4℃. On the following day, the sections were washed in 0.1 M PBS, and the secondary antibody was applied overnight at 4℃. The sections were then washed twice in 0.1 M PBS for 10 min and subsequently dried. Slides were viewed under a light microscope and muscle fibers were classified as either slow myosin heavy chain (MHC I; black) or fast myosin heavy chain (MHC II; green).

Western Blotting Analysis

Frozen muscle powder was homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 10% glycerol, protease inhibitor (Roche Applied Science), and phosphatase inhibitor (Thermo Scientific), with sonication for 10 s. Following this, the homogenate was centrifuged for 10 min at 14,000 ×*g,* at 4℃, after which theprotein extract was mixed with Laemmli sample buffer and then heated to 85℃. Samples were stored at - 80℃ until use. Equal amounts were loaded onto a gel for

electrophoresis, after which the separated proteins were electroblotted onto a nitrocellulose membrane (GE Healthcare) for 90 min.

After blotting, the membrane was washed in Tris-buffered saline containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (TBS-T), blocked with 1% skimmed milk (BD Pharmagen), and then incubated with the desired with the primary antibody overnight at 4℃. On the next day, the membrane was washed with TBS-T, and peroxidase-conjugated secondary antibodies (Thermo scientific) were applied for 60 min at room temperature. Chemiluminescent reagents (Thermo scientific) were used for the signal detection. The primary antibodies used in this study were phospho-p70S6K (Thr389), phospho-ERK1/2 (Thr202/Tyr204), and α-tubulin (all from Cell Signaling Technology. Japan). Images were then captured, and the signals were quantified using the Ez-capture chemiluminescence detector (ATTO) and CS analyzer software (ATTO). All target proteins were normalized α-tubulin expression levels.

Myofibrillar Protein Extraction

Procedures to determine the myofibrillar protein concentration were carried out as described by Karagounis et al (59). Approximately 20 mg of powdered muscle was homogenized in a buffer containing 150 mM NaCl, 0.1% Triton-X 100, 20mM Tris-HCl (pH 6.8), 50 μM dithiothreitol (DTT), 100 mM EDTA, and protease inhibitor (Roche Applied Science) and then centrifuged at 1,600 ×*g* for

20 min to produce a myofibrillar pellet. Following removal of the supernatant, the pellet was washed in a low-salt buffer containing 100 mM KCl, 5 mM Tris-HCl (pH 7.4), and 1 mM DTT and centrifuged for 5 min at 13,000 ×*g*; the process was repeated twice. The pellet was then washed 2 times in 70% ethanol. After removal of the ethanol, the pellet was resuspended in 0.3 M NaOH, and an aliquot was removed to determine the protein content by using the Lowry assay (RC DC Assay; BioRad). Protein concentrations were corrected for their bovine serum albumin content.

Cross-sectional Area measurement

To investigate the cross-sectional area (CSA), I used the immunohistochemical technique described by Bloemberg et al (60). The primary antibodies used were BA-F8 (MHC I), SC-71 (MHC IIa), BF-35 (MHC IIx), and BF-F3 (MHC IIb), all purchased from the Developmental Studies Hybridoma Bank (University of Iowa). The CSA quantification was performed by a computer application (Winroof; Mitsuya Co., Ltd.), with n=4 in each group. The CSA of each MHC isoform was counted to 100 fibers per sample.

Electrophoresis for Myosin Heavy Chain Composition

The electrophoretic protocol for separation of the myosin heavy chain isoforms was essentially the same as that described by Mizunoya et al (61). Muscle powder was homogenized in a buffer of

10% sodium dodecyl sulfate (SDS), 40 mM DTT, 5 mM EDTA, 100 mM Tris-HCl (pH 8.0), and protease inhibitor (Roche Applied Science). The homogenates were centrifuged at 15,490 ×*g* for 5 min at 4℃. The supernatant was extracted, and the protein concentration for each sample was determined by ultraviolet absorption spectrophotometry.

Samples were diluted to final protein concentrations of 10-1,280 ng/μL in a mixed sample buffer containing 100 mM DTT, 4.0% SDS, 160 mM Tris-HCl (pH 6.8), 43% glycerol, 0.2% bromophenol blue, and dH_2O. After boiling, the protein concentrations were adjusted to 20 ng/μL and frozen at -80℃ until use.

The separating gel consisted of 30% glycerol and 8% acrylamide, and the stacking gel consisted of 30% glycerol and 4% acrylamide. Electrophoresis was performed at 4℃ with a constant voltage of 140 V for 22 h (except for the first 40 min, during which the maximal current was limited to 10 mA to allow stacking gel penetration). The lower running buffer was mixed gently with a magnetic stirrer throughout the entire electrophoresis. After electrophoresis, the gels were stained with a silver staining kit (Silver Stain KANTO III: Kanto Chemicals), and then dried using a Multi Gel Dryer (Cosmo Bio Co., Ltd.). Finally, the bands were quantified by densitometry (Cs Analyzer; ATTO).

Statistical Analysis

The statistical differences between the trained (T) and untrained (UT) legs were determined by

paired *t*-tests. Concurrently, the statistical differences between HFS and LFS treatments were determined by unpaired *t*-tests. The time course changes in protein expression were examined by a two-way analysis of variance. A post hoc Bonferroni correction was performed using the *t*-test, and all numbers are expressed as the mean ± SD. $P < 0.05$ was considered to denote acceptable significance.

Results

Experiment I: One Bout of Exercise Induced by NMES with Low and high Electrical Frequencies

Mechanical Parameter of Force Generation

Figure 2-2 indicates the recorded parameter of force generation from 1 bout of training in the Post0 and Post3 groups. Figure 2A represents the typical schema of force generation in 1 bout of training. The top figure represents the HFS group and the bottom figure represents LFS group. Figure 2B represents the peak torque (Po), and Figure 2C indicates the force integral (mNm.S). Both the Po and force integral in the LFS group were significantly lower than the values in the HFS group ($P < 0.001$).

PAS Staining and Immunohistochemistry

Figure 2-3 shows the PAS and immunohistochemical staining results for type II fibers of rat skeletal muscle. Figure 2-3A suggests that type II fibers were dominant in the white portion. On the other hand, Figure 2-3B indicates that both type I and type II fibers co-existed in the red portion.

In Figure 2-3A, weak PAS staining was observed in the entire region, suggesting that glycogen depletion occurred under both HFS and LFS. The PAS staining in Figure 3B shows weakly stained

muscle fibers occurring randomly in type I fibers and type II fibers in both high-frequency- and low-frequency-stimulated muscles, suggesting that electrical stimulation recruited both type I and type II fibers, regardless of the frequency.

Time-Course Changes of Protein Expression

Figure 2-4 surveys the change in protein expression over different time points. Figure 2-4A shows results of the western blot analysis for the state of p70S6K phosphorylation at Thr 389. At both Post0 and Post3, HFS resulted in higher phosphorylation levels than in the UT ($P < 0.01$ at Post0, $P < 0.001$ at Post3; Post0 vs. Post3 $P < 0.05$). However, LFS caused no significant variation over either time point. Moreover, the phosphorylation of p70S6K in the LFS group at Post0 was significantly lower than that attributed to HFS at Post0 ($P < 0.05$).

Figure 2-4B represents the state of ERK 1/2 phosphorylation at Thr202/Tyr204. Following HFS, the phosphorylation levels at Post0 and Post3 remained unchanged. However, at Post0, only LFS training produced significantly higher levels of phosphorylation ($P < 0.05$).

Experiment II: Six Sessions of Training with NMES at Differential Frequencies

Physiological Characteristics after Six Sessions of Training Employing Low and high Electrical

Frequencies

Table 2-1 summarizes the results observed after 6 sessions of training with NMES at differential frequencies. No significant change in body weight occurred between the rats treated with HFS and LFS. However, both the HFS and LFS groups exhibited significant increases in both muscle weight (HFS: $P < 0.01$; LFS: $P < 0.01$) and myofibrillar protein content compared with the UT group(HFS: $P < 0.01$; LFS: $P < 0.05$). More importantly, there was no marked difference in these physiological characteristics between the HFS and LFS groups.

Myosin Heavy Chain Composition after Six Sessions of Training

Figure 2-5 indicates the composition of myosin heavy chains after 6 sessions of training with NMES at differential frequencies. Figure 2-5A shows that HFS significantly decreased the expressions of MHC II b ($P < 0.05$) and MHC I ($P< 0.05$) and significantly increased the expression of MHC IIx ($P < 0.01$). In contrast, Figure 2-5B reveals that LFS did not change the MHC composition.

Cross-sectional Area Measurement

Figure 2-6 represents the CSA measurements of the MHC isoforms. Figure 2-6A represents the HFS group and Figure 2-6B shows the LFS group. No significant change in the CSA of all MHC

isoforms was evident in both the HFS and LFS groups.

Discussion

In this study, I found that 1 bout of intermittent low-frequency electrical stimulations successfully activated one of the anabolic responses of ERK signaling. I also found that chronic bouts of LFS significantly increased the muscle mass and myofibrillar protein. This increase was comparable to that obtained with HFS, which has already been shown as training for muscle hypertrophy. I also found that MHCs and activated signaling molecules differed in composition in LFS- and HFS-induced hypertrophies. In the following discussion, I will discuss the potential biological mechanisms underlying LFS-induced muscle hypertrophy.

A number of studies have demonstrated that, the level of p70S6K phosphorylation can be used as an indicator of muscle hypertrophy and/or anabolic response. Our examination found that a significant increase in phosphorylated p70S6K was only observed in the HFS group. Mitchell et al. (11) reported that the level of p70S6K phosphorylation does not increase during low-intensity combined with high-volume training; yet our data showed skeletal muscle hypertrophy occurring with LFS without the phosphorylation of p70S6K. This may be explained by the possibility that our experimental model of LFS represents this low-intensity and high-volume training condition. I also investigated the phosphorylation level of ERK 1/2, a member of the MAPK signaling pathway, and found it to be increased only in the LFS group, immediately after training. Since the phosphorylation of p70S6K is elicited mainly in type II fibers (39, 62), the activation of type II fibers and the increase

in MHC IIx might be related to signal transduction during HFS. As ERK 1/2 expression did not demonstrate fiber type specificity, our observation that the MHC composition was not affected by LFS stimulation suggests that a relatively high activation of ERK 1/2 might lead to unspecific hypertrophy in rat medial gastrocnemius muscles. Taken together, I speculate that mTOR signaling is a major contributor to HFS-induced hypertrophy, and that MAPK signaling plays a role in LFS-induced hypertrophy.

A key finding in the current study was that the 6-sessions trainings with LFS successfully induced a significant increase in muscle mass and myofibrillar protein contents, and that this increase was determined to be the same in the muscle stimulated with HFS. Since 1 bout of LFS exercise induces ERK phosphorylation, chronic bouts of LFS is an accumulation of such acute anabolic response. However, I failed to observe a significant increase in the CSA analysis after chronic bouts of training. This may be ascribed to the fact that 6 sessions of training is a comparatively shorter time than previous studies (56, 63).

In the Introduction section, I raised the possibility that NMES randomly activates type I and type II fibers, leading to muscle hypertrophy. In this study, I performed PAS-staining to investigate which fibers were activated by NMES. The results showed that muscle fibers were randomly activated by both LFS and HFS, independent of the exerted force. The manner of muscle fiber activation under electrical stimulation was similar to that of a previous report, suggesting that NMES activates

muscle fibers at random (54). Taken together, I consider that both type I and type II fibers are activated under both HFS and LFS treatmens.

In both the LFS and HFS groups, the intramuscular glycogen was mainly depleted in the fibers located in the white portion in the medial gastrocnemius muscle. In the red portion, intramuscular glycogen was depleted in the HFS group, but clear staining was observed after LFS treatment. The HFS protocol in our experiment is essentially the same as that used by previous studies (21, 24, 63), and this condition led to a supramaximal activation of gastrocnemius muscles. On the other hand, I observed that the exerted force and work volume in the LFS group was about less than 50% of that in the HFS group. Such differences in exerted force could be related to differences in glycogen consumption.

As seen in the immunostaining of type II fibers, the gastrocnemius muscle consisted mainly of type II fibers, especially in the white portion. This is the same result as shown by previous studies (56, 60, 63). Since the major fibers were of type II in the white portion, random activation dominantly recruited type II fibers, regardless of the electrical frequency. Since type II fiber activation is a key event in muscle hypertrophy, LFS might successfully induce muscle hypertrophy in gastrocnemius muscles.

Recently, muscle hypertrophy was reported to be induced by moderate- and/or low-intensity (20-50% of 1 RM) resistance training, with blood-flow restriction (64-67). Moreover, Fujita et al.

(68) have also reported that an increase of the muscle protein synthesis rate occurs in the 3 h following low-intensity, blood-flow-restricted resistance exercise. More recent studies have determined that low intensity training to the point of fatigue without blood-flow restriction also elicits a higher protein synthesis rate and muscle hypertrophy (10, 11). This supports the idea that a recruitment of type II fibers occurs under fatigable conditions, even if the weight lifted is low. It is obvious that human studies are far from the animal models, and our LFS model underscores the theory that activation of type II fibers is a key factor in achieving muscle hypertrophy without high exerted force.

Various exercise methods can induce fiber type alteration. I examined whether training could stimulate fiber type changes, by modifying the myosin heavy chain composition. As previously reported, during HFS, MHC IIx expression was increased in the medial gastrocnemius muscle, whereas MHC IIb and MHC I expressions were decreased (56, 63). Unexpectedly, I found that LFS did not change the MHC composition. Normally, ATP-consuming muscle contraction induces the switch from a fast- to a slow-twitch fiber activation. Muscle hypertrophy, with no change in the MHC composition, suggests that all muscle fibers are equally enlarged, independent of fiber type. I also speculate that the groups of activated signaling molecules might differ between LFS and HFS. In the acute experiment, I found that p70S6K was activated by HFS, and ERK 1/2 was activated by LFS. Since the phosphorylation of p70S6K elicited mainly type II fibers (39, 62), the activation of

type II fibers and the increase in MHC IIx might be related to the signal transduction induced by HFS. As ERK 1/2 expression did not demonstrate fiber type specificity, our observation that the MHC composition was not affected by LFS suggests that a relatively high activation of ERK 1/2 might lead to unspecific hypertrophy in the rat medial gastrocnemius muscle. It is also important to evaluate whether functional differences exist between muscles treated with different electrical frequencies.

For humans, skeletal muscle activation by NMES is a method of physical therapy aimed at augmenting and/or maintaining skeletal muscle performance. In clinical settings, high-frequency NMES is mostly dedicated to the improvement of the muscular strength and is similar to resistance training (69). Conversely, low-frequency NMES improves the metabolic and histochemical characteristics of the skeletal muscle and is thought to mimic endurance training (70). Our data suggest that electrical stimulation of the skeletal muscle with low-force generation can be beneficial in achieving muscle hypertrophy without the pain associated with high frequency electrical stimulation.

In summary, our present study demonstrates that muscle activation by electrical stimulation recruits type II fibers independently of frequency, and that electrical stimulation without high force generation results in muscle hypertrophy. This finding may be applicable to both athletic conditioning as well as to clinical care for sports injuries and muscle atrophy.

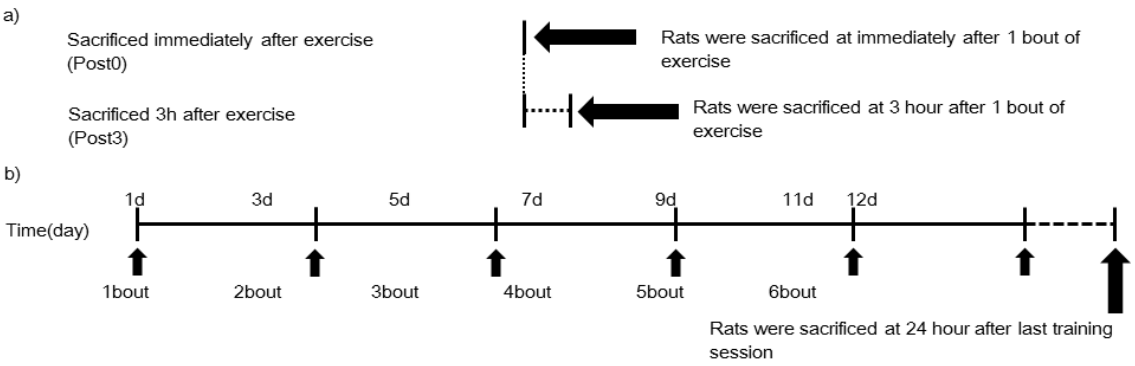

FIGURE 2-1. Training protocol

Schematic diagram representing the details of the experimental schedule in this study. Thirty-six Sprague-Dawley rats were used. The rats were divided into 2 groups: high-frequency electrical stimulation (HFS) and low-frequency electrical stimulation (LFS). Rats were subjected to training on every other day. (a) The upper illustration represents 1 bout of exercise. (b) The lower illustration represents 6 sessions of a training protocol. The rats in all of the experimental groups (n = 6) had their medial gastrocnemius muscle extracted after death.

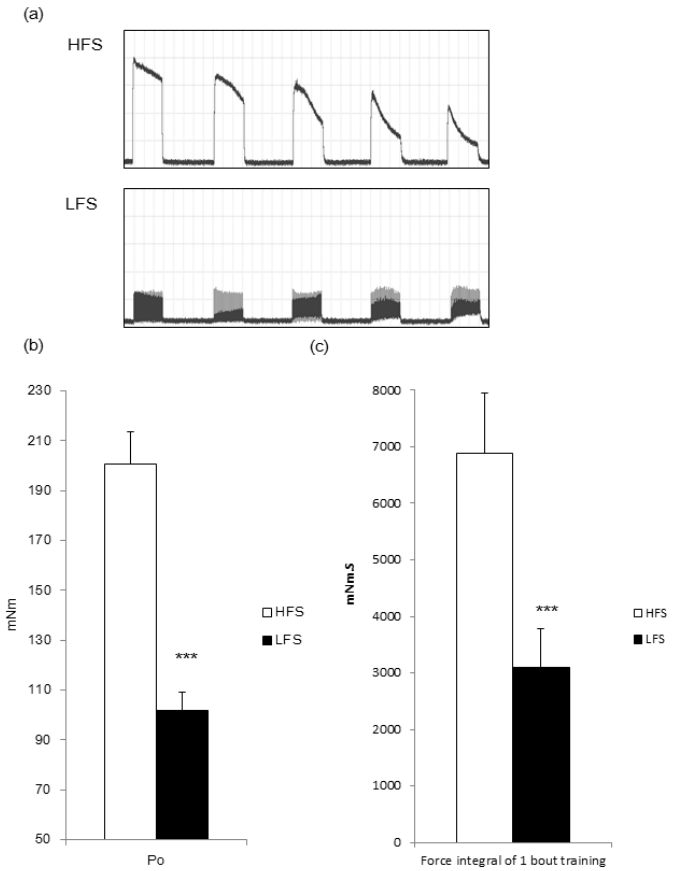

FIGURE 2-2. Mechanical parameter of force generation

(a) The typical schema of force generation of each training group. The top figure represents high-frequency electrical stimulation (HFS) and the bottom figure shows low-frequency electrical stimulation (LFS). (b) The peak tetanic torque (Po). (c) The total force generation of 1 bout of exercise. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. All values are the mean ± SD. *** $P < 0.001$, vs. HFS.

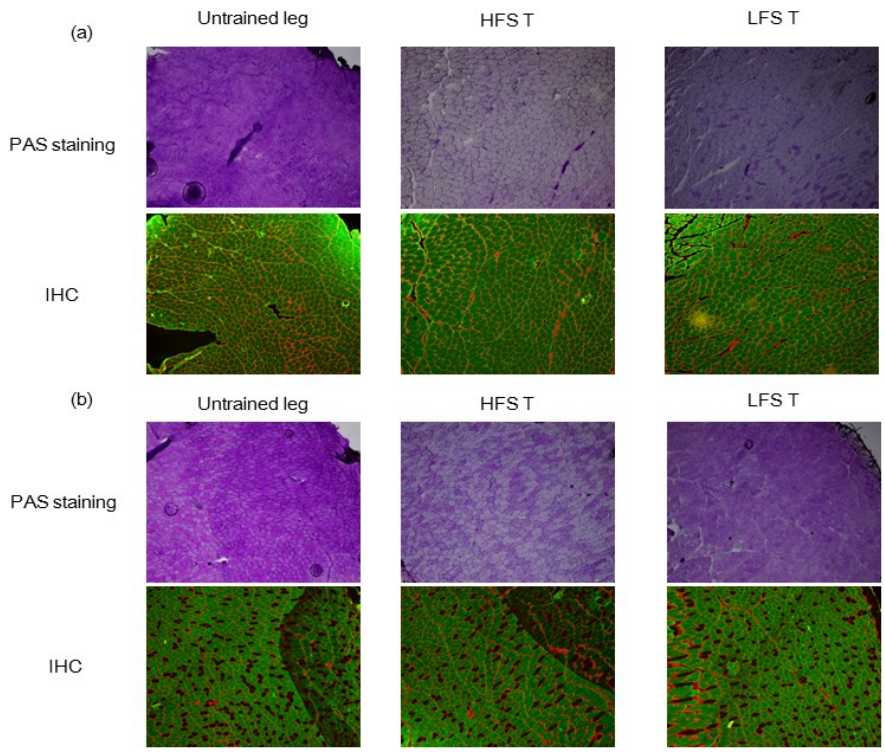

FIGURE 2-3. PAS staining and immunohistochemistry

(a) PAS and immunohistochemistry staining patterns in the white portion of the medial gastrocnemius. (b) PAS and immunohistochemistry staining patterns in red portion of the medial gastrocnemius. In PAS staining, the intramuscular glycogen content is indicated by a deep purple color. In immunohistochemistry, myosin heavy chain I (black) and II (green) are indicated. IHC: imunohistochemistry. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. HFS T and LFS T: the trained leg in HFS and LFS, respectively.

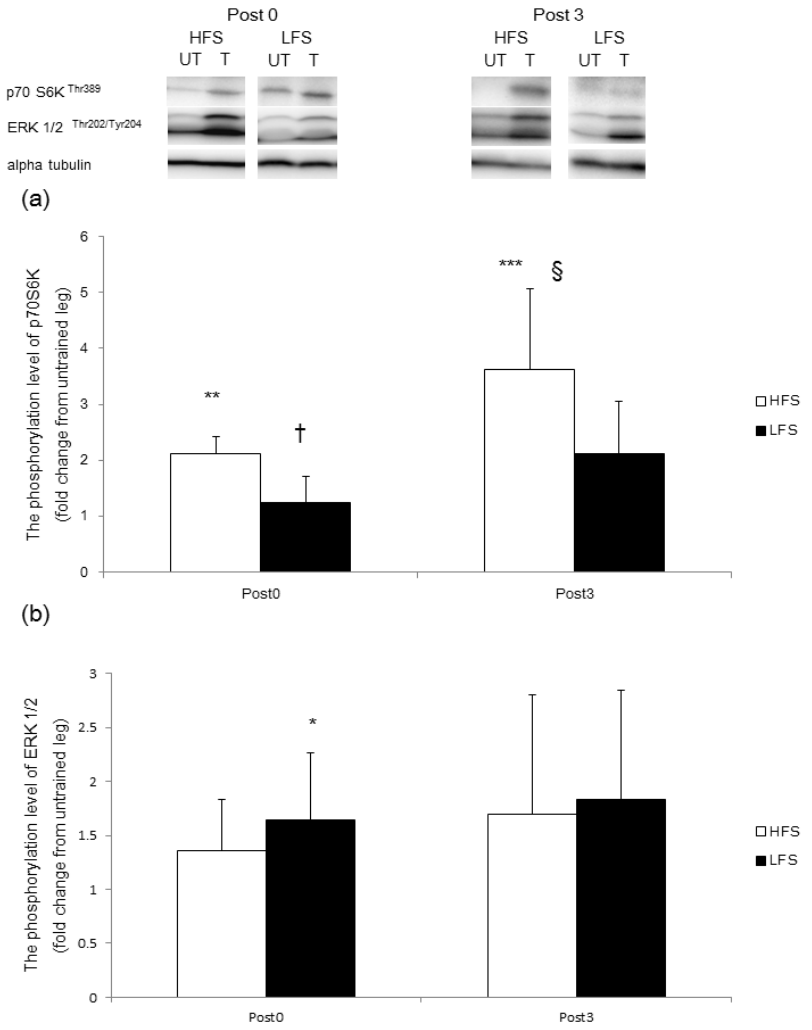

FIGURE 2-4. Time-course changes of protein expression

(a) The level of p70S6K phosphorylation at Thr389. (b) The level of ERK 1/2 phosphorylation at Thr202/ Thr204. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. UT: the untrained leg. The relative value was defined as the ratio of trained leg against untrained control leg. All values are the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, vs. UT; $^{\dagger}P < 0.05$, vs. HFS; $^{\S}P < 0.05$, vs. Post0.

Table 2-1. Physiological characteristics after six sessions of training employing Low and high electrical frequencies.

		HFS	LFS
Body weight (g)		366.36±22.17	362.31±12.36
Medial gastrocnemius wet weight (mg)	T	887.10±20.77 **	883.92±37.50 ***
	UT	832.02±35.54	823.08±37.48
Medial gastrocnemius wet weight /Body weight	T	2.43±0.13 **	2.44±0.09 ***
	UT	2.28±0.11	2.27±0.05
% increase of muscle wet weight (T vs. UT)		6.78±5.14	7.44±3.07
% increase of myofibrillar protein content (T vs. UT)		21.67±13.62 **	23.80±15.89 *

HFS: high frequency electrical stimulation, LFS: low frequency electrical stimulation, T: trained leg , and UT: untrained leg. Values are mean ±SD. * p< 0.05 , ** p<0.01, *** p<0.001 vs. UT.

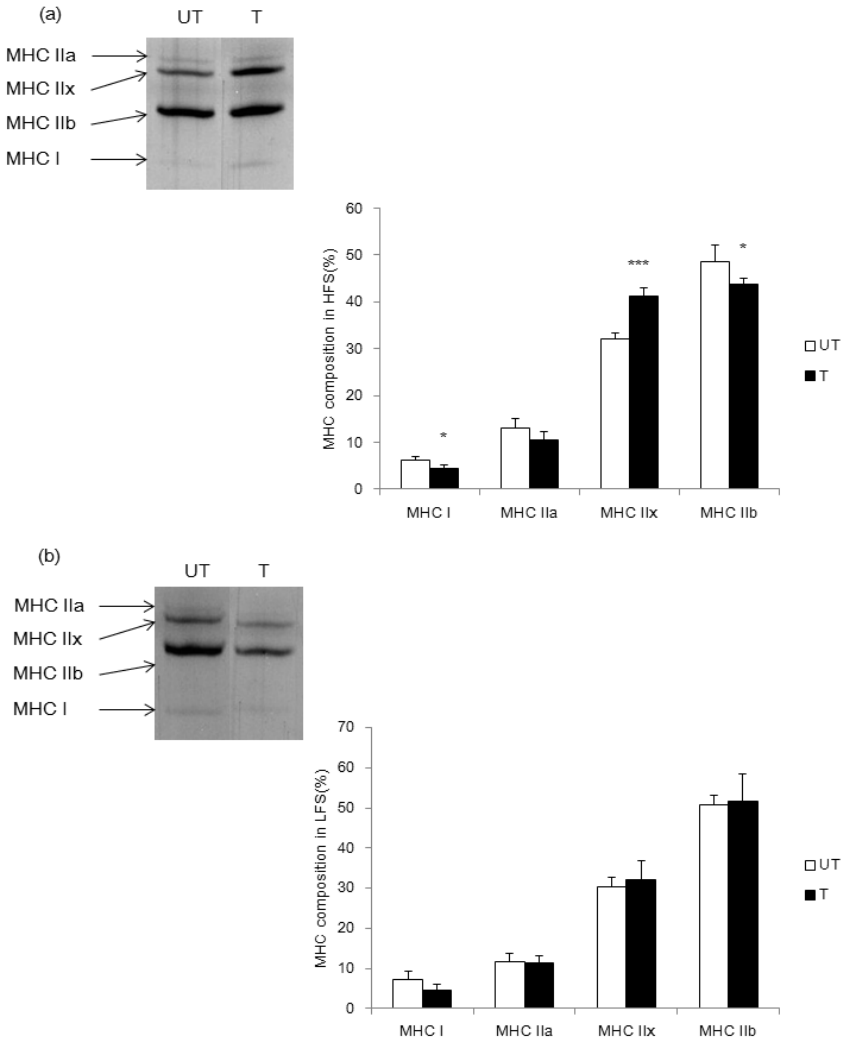

FIGURE 2-5. Myosin heavy chain composition after 6 sessions of training

(a) Myosin heavy chain composition after 6 sessions of training in HFS. (b) Myosin heavy chain composition after 6 sessions of training in LFS. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. T: the trained leg. UT: the untrained leg. MHC: myosin heavy chain. All values are the mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$, vs. UT.

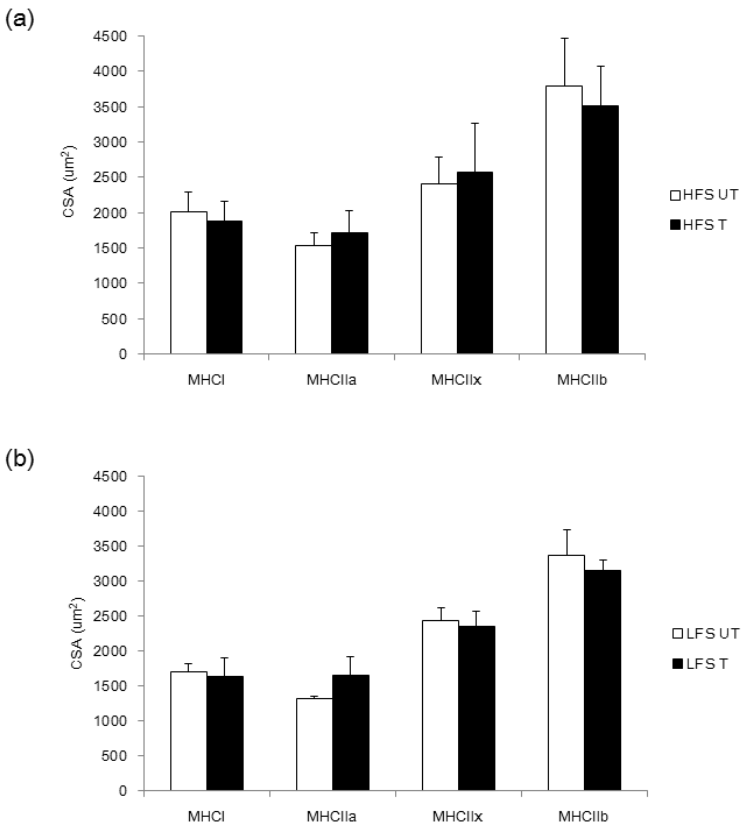

FIGURE 2-6. CSA measurement

(a) The CSA after 6 sessions of training in HFS. (b) The CSA after 6 sessions of training in LFS.

HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group.

T: the trained leg. UT: the untrained leg. CSA: cross-sectional area. All values are the mean ± SD.

Chapter 3

The acute molecular events for protein synthesis after intermittent low-frequency electrical stimulation in rat gastrocnemius muscle

Introduction

Muscle contractile activity leads to the potential for increasing mass, termed hypertrophy. Especially, resistance training with high force production is recommended for skeletal muscle hypertrophy (4). Muscle hypertrophy is occurred by increasing of muscle protein synthesis (MPS) before, during and after acute bout exercise (71, 72). Recent studies have reported that low loading condition combined high repetition resistance training also induces skeletal muscle hypertrophy (10, 11). Burd et al. has reported that higher MPS of low-loading with high repetition exercise continues to maintained in comparison with MPS of high-loading with low repetition exercise (10). Thus, it might be that anabolic response induced by low-loading with high repetitive contractions will be differ from high-loading with low repetition stimulus.

Increasing of MPS after exercise is regulated by intracellular signaling (73). The signaling networks controlling protein synthesis through translation initiation involve phosphorylation of the mammalian target of rapamycin complex 1 (mTORC1) and sequential activation of p70 KDa ribosomal protein S6 kinase (p70S6K). The mTORC1 has thought a critical regulator for increasing MPS, cell growth, and controlling translation (74, 75). It has thought that resistance training induced hypertrophy by high intensity loading is also regulated by mTORC1 signaling (76, 77). Co-instantaneously, p70S6K is well investigated signaling molecule. Previous studies have reported that phosphorylation level of p70S6K is correlated with skeletal muscle mass (39, 78). The

Threonine 389 residue of p70S6K is phosphorylated by mTOR (74). On the other hand, mitogen activated protein kinase (MAPK) pathway is also known the regulator of increasing MPS by mechanical loading (40, 52). Extracellular signal regulate kinase (ERK) 1/2, one of the MAPK molecule, is also implicated in increasing MPS. Moreover, ERK 1/2 phosphorylation also phosphorylate p70S6K at other phosphorylating site i.e. Serine 421/ Threonine 424 (79). These two pathways have shared downstream substrate, ribosomal protein S6 (rpS6), which is also implicated cell growth (43, 80). The phosphorylated p70S6K can promote phosphorylate of rpS6 at residue of Serine 240/244 (81). Although above network has been well-investigated in high intensity exercise-induced MPS, hypertrophic response under low force generating condition is still unclear.

Neuromuscular electrical stimulation (NMES) with high frequency on rat skeletal muscle is one animal experimental model to mimic high intensity resistance training for leading skeletal muscle hypertrophy (11, 55). On the other hand, our previous study has reported intermittent low frequency NMES (LFS) succeeded leading the increase of muscle mass and myofibrillar protein content (20). In this paper, we also found phosphorylation level of p70S6K was not increased at 3h post exercise (20). Thus, in LFS-induced skeletal muscle hypertrophy, it might be that responsive skeletal muscle hypertrophy related pathway is different from commonly accepting resistance training with high force contraction.

In present study, I aimed to investigate whether LFS induced muscle anabolism is regulated via

mTORC1. Therefore, we focused on phosphorylation status of each molecule i.e. mTOR (S2448), ERK 1/2 (T202/Y204), p70 S6K (T389 and T421/S424), and rpS6 (S240/244). I hypothesized that LFS-induced MPS is implicated MAPK rather than mTOR pathway.

Materials and Methods

Animal care

All experimental procedures performed in this study were approved by the Ethics committee for animal experiment at Nippon Sports Science University. Male Sprague Dawley rats, 9 weeks of age, were used in this study. Animals were housed in temperature and humidity controlled holding facilities on a 12 light-dark cycle and had access to food and water *ad libitum*.

Exercise Protocol

Under anesthesia, the right lower leg of each rat was shaved. The rats were then subjected to isometric training by electrical stimulation. Each rat was laid prone on a platform and its right knee was extended with a dynamometer, with the ankle joint positioned at an angle of 90°. The triceps of the right leg muscle was then stimulated (voltage: 30-35 V; pulse duration 4 ms: frequency: 100 Hz for HFS group and 10 Hz for LFS group) with a surface electrode (7.5 mm × 7.5 mm) that was connected to an electrical stimulator and isolator (Nihon Koden, Japan). The left medial gastrocnemius muscle served as the untrained (UT) control.

For all exercise groups, the triceps surae muscle was trained by stimulation for five 3s contractions, with a 5s interval between each contraction. Four sets in total were performed, with 3

min intervals between each set. After 1 bout of training session, the food was deprived until sacrifice.

Animals were sacrificed immediately, 3, 12, and 24 h after exercise (n=6: each group). The medial gastrocnemius muscle was removed, weighed, and rapidly frozen in liquid nitrogen, and the right and left medial gastrocnemius muscles were triturated in liquid nitrogen and stored at – 80℃ until use.

Western blot analysis

Frozen tissues were sonicated for 10 s in ice-cold RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 25mM Tris-Hcl pH 7.6, 150mM NaCl, 5mM EDTA, 1% NP-40, 1% sodium deoxycholate, and protease and phosphatase inhibitor (Thermo scientific). Homogenate were then centrifuged 15,000×*g* for 20 min at 4℃ and supernatant collected for analysis. Sample protein concentrations were determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts (6.7μg) of protein from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on TGX gels (Bio-Rad Laboratories, Hercules, CA, USA). After end of gel running, separate proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat dry milk in TBS-T (Tris-bufferd saline mixed with 0.1% Tween 20) for 60 min followed by an overnight incubation at 4℃ with dilutions of each primary antibodies dissolved in TBS-T containing 5%

(except phosphorylation of p70S6K at T389) and 5% non-fat dry milk (phosphorylation of p70S6K1 at T389). Membranes were washed for 15 min in TBS-T and then incubated for 1 h at room temperature in 5% non-fat dry milk TBS-T containing horseradish peroxidase conjugated secondary antibodies. After 15 min of washing in TBS-T, the blots were detected using chemiluminescence reagent (West femto maximum, Thermo scientific). Protein expression was measured by computer application (CS analyzer, ATTO).

mTOR (phospho and total), ERK 1/2 (phospho and total), rpS6 (phospho and total), and p70S6K (phospho at T421/S424 and total) primary antibodies were purchased from Cell Signaling technology. Phosphorylation of p70S6K at T389 antibody was purchased from Santa Cruz Biotechnology.

Statistical Analysis

The statistical differences between the trained (T) and untrained (UT) legs were determined by paired *t*-tests. The time course changes and between HFS and LFS in protein expression were examined by a two-way analysis of variance. A post hoc Bonferroni correction was performed using the *t*-test, and all numbers are expressed as the mean ± SD. $P < 0.05$ was considered to denote acceptable significance.

Results

Time course changes of p70S6K

I) The comparison of T leg with UT leg in each group and the comparison between HFS and LFS

Figure 3-1A and 3-1B represents phosphorylation status of p70S6K at T389 and T421/S424 residues, respectively. Figure 3-1A shows that the phosphorylated level of p70S6K at T389 in HFS T was higher than HFS UT at Post 0 ($p<0.05$) and Post 3 ($p<0.05$) and there was significantly differences between HFS higher than LFS at Post 3 ($p<0.05$). The phosphorylation level of p70S6K at T389 in LFS T was only higher than in LFS UT at Post 12 ($p<0.05$) and there was significantly differences between LFS higher than HFS ($p<0.05$).

Figure 3-1B shows that phosphorylated level of p70S6K at T421/S424 in HFS T was higher than in HFS UT at Post 0 ($p<0.05$) and Post 3 ($p<0.05$) and there was significance between HFS higher than LFS at Post 3 ($p<0.05$). Phosphorylated level of p70S6K at T421/S424 in LFS T was higher than in LFS UT at Post 0 ($p<0.05$), Post 12 ($p<0.05$) and Post 24 ($p<0.05$).

II) The comparison of time course effects within each group

Figure 3-1C and 3-1D shows that time course changes of phosphorylation status of p70S6K at T389 and T421/S424, respectively. Figure 3-1C reveals that phosphorylation of p70S6K at T389 in

HFS at Post 3 was higher than at Post 0 ($p<0.05$). In LFS, phosphorylation status of p70S6K at T389 at Post 12 was higher than at Post 0.

Figure 3-1D discloses phosphorylation status of p70S6K at T421/S424 in HFS at Post 3 was higher than at Post 0, Post 12, and Post 24 ($p<0.05$). On the other hands, there were no significant differences in each group in LFS.

Time course changes of rpS6

I) The comparison of T leg with UT leg in each group and the comparison between HFS and LFS

Figure 3-2A shows the phosphorylation level of rpS6 at residue of S240/244. The phosphorylation level of rpS6 at S240/S244 in HFS T at Post 3, Post 12, and Post 24 were higher than HFS UT ($p<0.05$) and there was significantly difference in HFS higher than in LFS at Post 12 ($p<0.05$). The phosphorylation level of rpS6 at S240/244 in LFS T was higher than in LFS UT at only Post 3 ($p<0.05$).

II) The comparison of time course effects within each group

Figure 3-2B denotes that time course changes of phosphorylation status of rpS6 at S240/S244. The phosphorylation status of rpS6 at S240/244 in HFS at Post 3 was higher than at Post 0 and Post 24 ($p<0.05$) and at Post 12 was higher than at Post 0 ($p<0.05$). In LFS, the phosphorylation status of

rpS6 at S240/S244 at Post 3 was higher than Post 0, Post12, and Post 24 ($p<0.05$).

Time course changes of mTOR

I) The comparison of T leg with UT leg in each group and the comparison between HFS and LFS

Figure 3-3A shows the phosphorylation level of mTOR at residue of S2448. The phosphorylation level of mTOR at S2448 in HFS T was higher than HFS UT at Post 3 ($p<0.05$) and mTOR phosphorylation at S2448 in HFS at Post 3, Post 12, Post 24 were higher than in LFS ($p<0.05$). The phosphorylation level of mTOR at S2448 in LFS T was high than its UT at only Post0 ($p<0.05$).

II) The comparison of time course effects within each group

Figure 3-3B shows that time course changes of phosphorylation status of mTOR at S2448. There were no significant differences in each time point in HFS. The phosphorylation status of mTOR in LFS at Post 0 was higher than at Post3, Post12, and Post24 ($p<0.05$).

Time course changes of ERK1/2

I) The comparison of T leg with UT leg in each group and the comparison between HFS and LFS

Figure 3-4A represents the phosphorylation level of ERK 1/2 at residue of T202/Y204. There

were no significances between HFS T and HFS UT at all time point. The phosphorylation level of ERK 1/2 in LFS T was higher than in LFS UT at only Post 24 and there were no significances between HFS and LFS ($p<0.05$).

II) The comparison of time course effects within each group

Figure 3-4B shows that time course changes of phosphorylation status of ERK 1/2 at T202/Y204. In HFS, there were no significantly differences between each time point. The phosphorylation status of ERK 1/2 at T202/Y204 in LFS at Post 24 was higher than Post 0 ($p<0.05$).

Discussion

It is thought that muscle contractile activity to lead skeletal muscle hypertrophy is regulated by increasing MPS after acute bout of exercise. Our previous study has shown that LFS-induced hypertrophy rate is same to HFS (20). Thus, it is thought that increasing MPS by acute bout of exercise in LFS and HFS is also similar extent. In this study, I examined to investigate whether LFS induced MPS is regulated by mTORC1 signaling pathway. Although, I found that LFS induced MPS was mediated by at least p70S6K, it is possible that p70S6K phosphorylation is not mediated through generally accepting upstream molecule of p70S6K such as mTOR and ERK1/2. In this section, I try to discuss that how LFS induce high MPS after acute bout of exercise.

To date, it has been thought that mTOR and downstream target (mTOR pathway) is a critical regulator for up-regulating protein synthesis, cell growth, and translational control (38, 73, 74, 78). Rahnert and Burkholder (44) examined whether high frequency electrical stimulation promotes metabolic signal such as mTOR pathway and MAPK by using exenterate muscle. They reported that the phosphorylation of mTOR was correlated with total force amount i.e. force-time integral. Hulmi and coworkers (82) have suggested that the phosphorylation of p70S6K and rpS6 were increased according with exercise load increment in human resistance exercise. Indeed, HFS, mimicked high intensity resistance exercise, promoted higher phosphorylation of mTOR relative to UT control leg at all time point in this study. Contrastingly, LFS did not phosphorylation status of mTOR in

comparison with contralateral control and with HFS. Since I showed that LFS was low force production compared with HFS in chapter 2 (20), it might be thought that high force production is needed to increase MPS through mTORC1 pathway.

On the other hand, MAPK family also regulates MPS (83, 84). Especially, ERK 1/2, which is one of MAPK families, is known as an another regulator for muscle protein synthesis (40, 52). In the present study, I found that HFS and LFS similarly increased phosphorylation of ERK 1/2 after 24 hour of one bout of exercise. However, significant difference was identified relative to UT at only Post 24 in LFS. It is also known that ERK 1/2 implicated in cell proliferation as other function (85). Burd et al. (10) have reported that ERK 1/2 was increased at 4h and 24 h after low-load with high volume resistance exercise. Concurrently, they investigated MyoD and Myogenin, which are a member of Myogenic regulatory factors (MRFs), involved with muscle satellite cell differentiation and proliferation, respectively (86, 87). From these experiments, they identified increasing expression of Myogenin mRNA after 24 hours of low-load combined high volume exercise. Myogenic satellite cell is also related to the acute exercise induced muscle protein synthetic response at later time point (87). Thus, it might be that function of ERK 1/2 was shifted protein synthesis to muscle satellite cell proliferation by time course (summarized in Figure 3-5). However, I did not investigate MRFs and satellite cell interaction in this study. I should analyze MRFs and satellite cell activation in immediate future.

Recent studies have shown that p70S6K is often used as one of the indicators for protein synthesis (39, 62). Notably, phosphorylation site of Threonine 389 and Threonine 421/ Serine 424 sites are regulated by mTOR and ERK 1/2, respectively (74, 79). In present study, phosphorylation of p70S6K at T389 was increased in both HFS and LFS. Baar and Esser (39) have reported that total phosphorylation level of p70S6K was highly increased at post 3-6 h after resistance exercise. Interestingly, phosphorylation time point was delayed in LFS (Post12) compared with HFS (Post3). In this study, I aimed to search critical anabolic pathway in LFS-induced MPS. Although the phosphorylation of mTOR in HFS was increased and maintained relative to HFS UT at each time point, phosphorylation of mTOR in LFS was lower level than in LFS UT and HFS at Post 3, Post 12, and Post 24. There have reported that phosphorylation of p70S6K at T389 can also phosphorylate mTOR at S2448 as feedback (74, 88). Thus, it is controversial whether phosphorylation of p70S6K at T389 in LFS was associated with increasing MPS. On the other hands, despite phosphorylation status of ERK 1/2 represented similar biphasic state in both HFS and LFS at the each time point, phosphorylation status of p70S6K at T421/S424 in HFS was higher than in LFS at Post 3. Taken together, mTOR and ERK 1/2 dynamics was disagreed with downstream target of phosphorylated p70S6K at T389 and T421/S424 in LFS.

To confirm activation of p70S6K, I investigated phosphorylation status of rpS6 which is also related with cell growth and translation initiation (43, 80). Especially, rpS6 at residue of S240/244

was phosphorylated by activation of p70S6K (81). The phosphorylation of rpS6 was highest in Post3 both HFS and LFS. However, phosphorylation status of rpS6 in HFS was higher than LFS in Post 12. It might be suggested that increasing MPS in LFS was faster than HFS (summarized Figure 3-5).

In summary, I try to clarify the molecular mechanisms of LFS induced skeletal muscle hypertrophy focusing on mTOR signaling in this study. mTOR signaling is strongly associated with skeletal muscle hypertrophy under high force generative condition such as HFS. However, the detail of molecular mechanism of LFS-induced MPS is still unclear. Recent studies have shown that other molecules also implicated skeletal muscle hypertrophy, such as Bone Morphogenetic Protein (BMP) signaling (89) and Type IV collagen (90). Therefore, I feel the necessity to further analysis focusing on entirely different molecules.

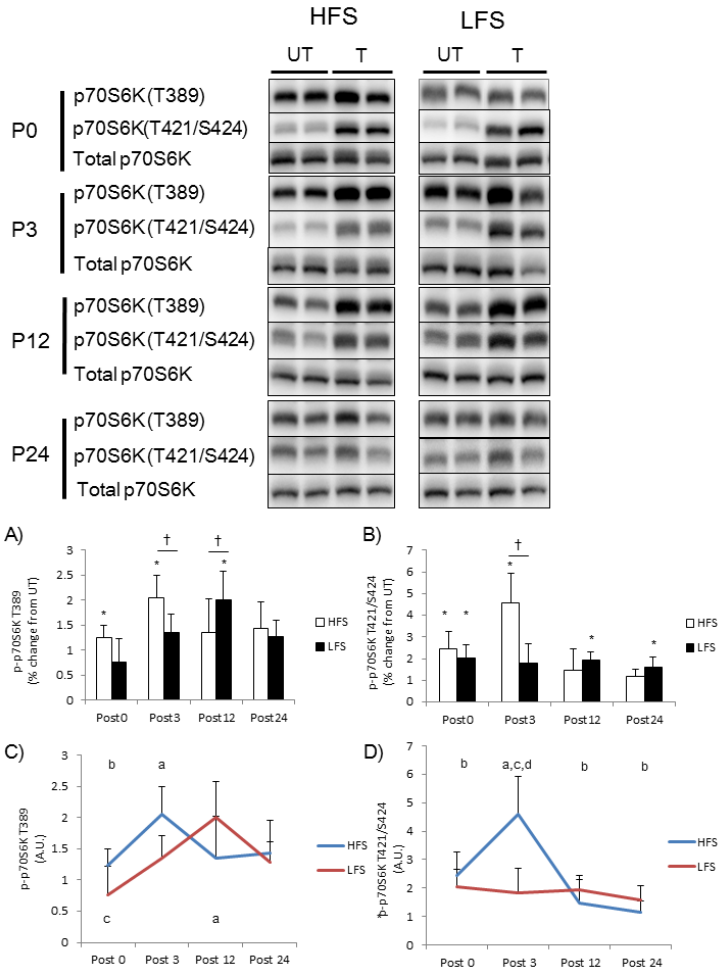

FIGURE 3-1. Time course changes of p70S6K

(A) and (B) The level of p70S6K phosphorylation at T389 and T421/S424, respectively. (C) and (D) The time course expression of p70S6K phosphorylation at T389 and T421/ S424, respectively. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. The relative value was defined as the ratio of trained leg against untrained control leg. All values are the mean ± SD. $^{*}P < 0.05$, vs. UT; $^{\dagger}P < 0.05$, vs. HFS at each time point; $^{a}P < 0.05$, vs. Post0, $^{b}P < 0.05$, vs. Post3, $^{c}P < 0.05$, vs. Post12. $^{d}P < 0.05$, vs. Post24.

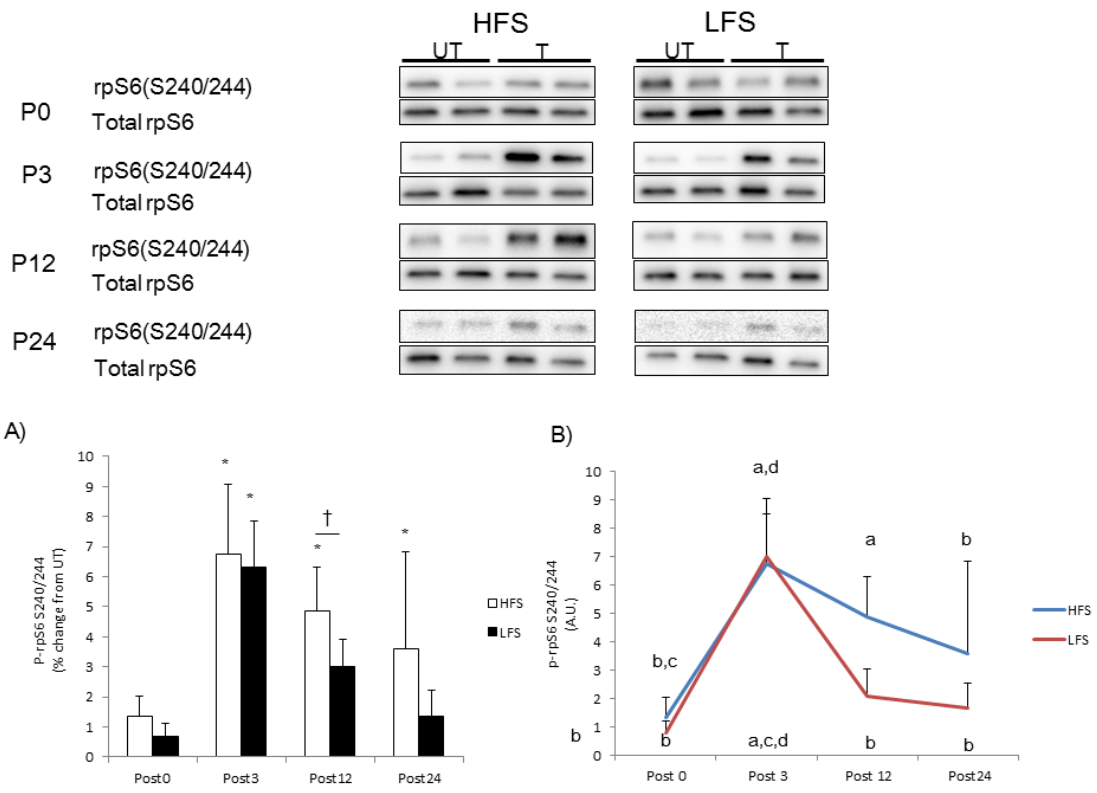

FIGURE 3-2. Time course changes of rpS6.

(A) The level of rpS6 phosphorylation at S240/244. (B) The time course expression of rpS6 phosphorylation at S240/244. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. The relative value was defined as the ratio of trained leg against untrained control leg. All values are the mean ± SD. $^{*}P < 0.05$, vs. UT; $^{\dagger}P < 0.05$, vs. HFS at each time point; $^{a}P < 0.05$, vs. Post0, $^{b}P < 0.05$, vs. Post3, $^{c}P < 0.05$, vs. Post12. $^{d}P < 0.05$, vs. Post24.

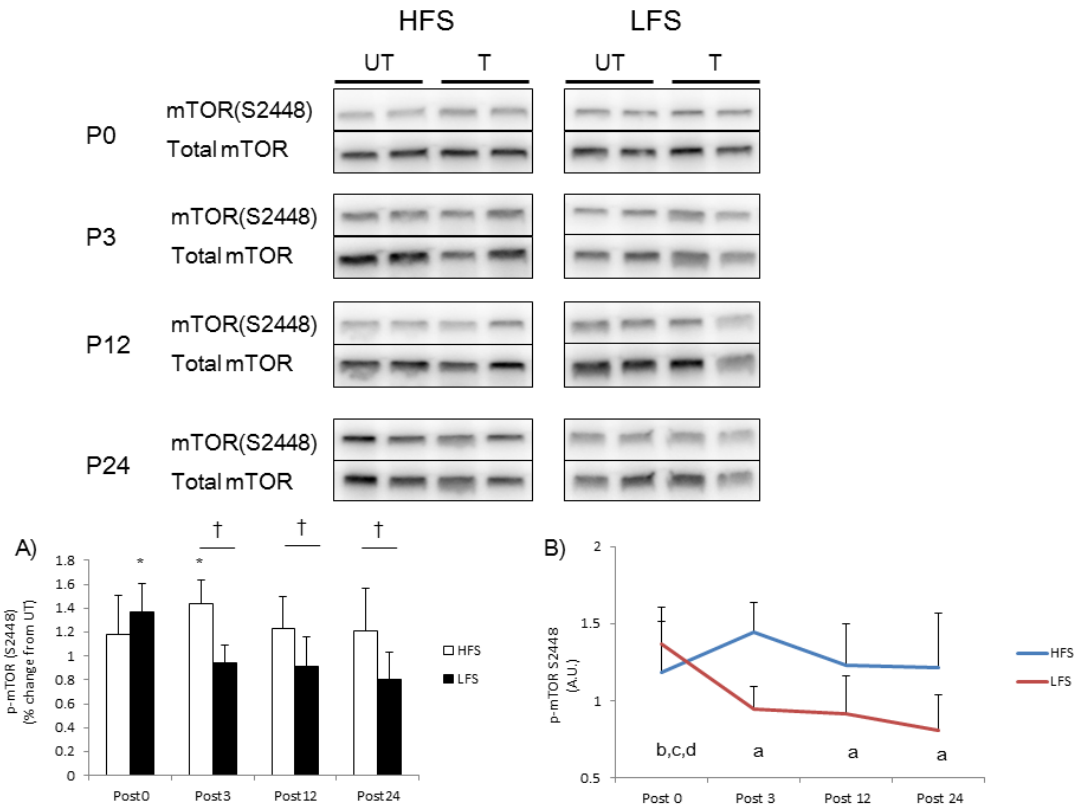

FIGURE 3-3. Time course changes of mTOR.

(A) The level of mTOR phosphorylation at S2448. (B) The time course expression of mTOR phosphorylation at S2448. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. The relative value was defined as the ratio of trained leg against untrained control leg. All values are the mean ± SD. $^{*}P < 0.05$, vs. UT; $^{\dagger}P < 0.05$, vs. HFS at each time point; $^{a}P < 0.05$, vs. Post0, $^{b}P < 0.05$, vs. Post3, $^{c}P < 0.05$, vs. Post12. $^{d}P < 0.05$, vs. Post24.

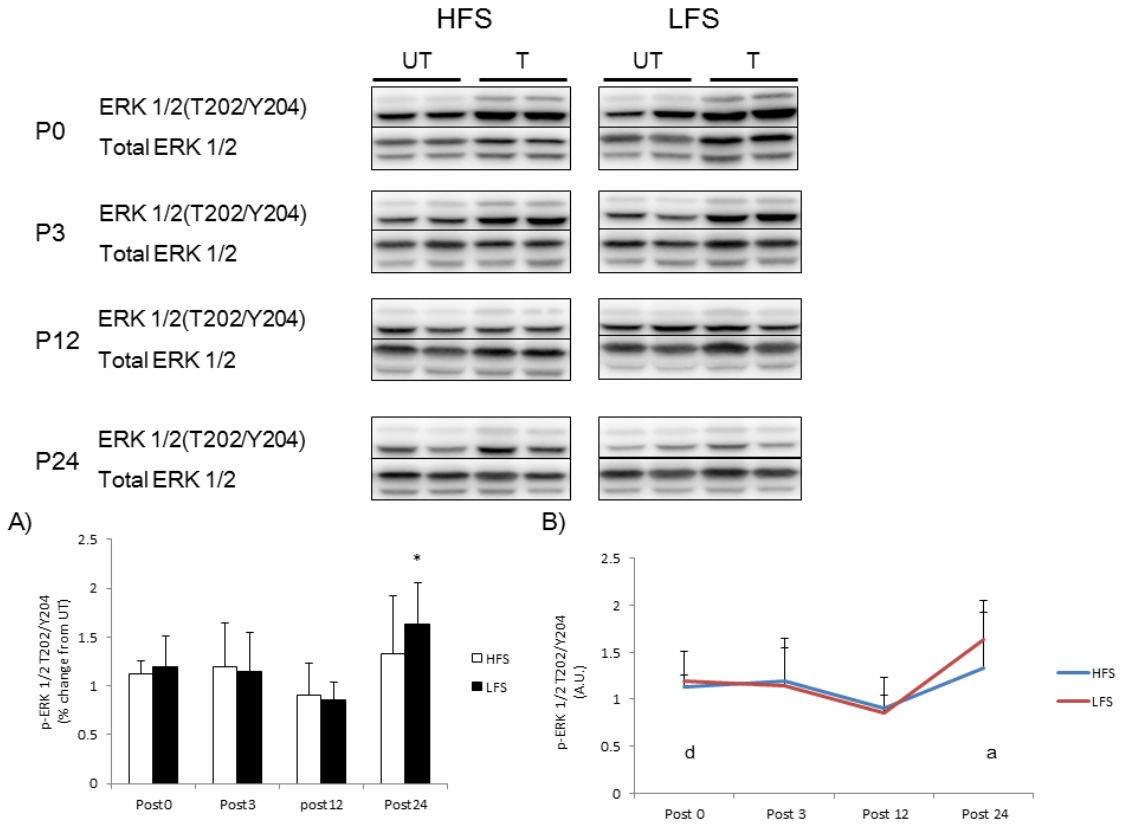

FIGURE 3-4. Time course changes of ERK 1/2.

(A) The level of ERK 1/2 phosphorylation atT202/Y204. (B) The time course expression of ERK phosphorylation at T202/Y204. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. The relative value was defined as the ratio of trained leg against untrained control leg. All values are the mean ± SD. $^{*}P < 0.05$, vs. UT; $^{\dagger}P < 0.05$, vs. HFS at each time point; $^{a}P < 0.05$, vs. Post0, $^{b}P < 0.05$, vs. Post3, $^{c}P < 0.05$, vs. Post12. $^{d}P < 0.05$, vs. Post24.

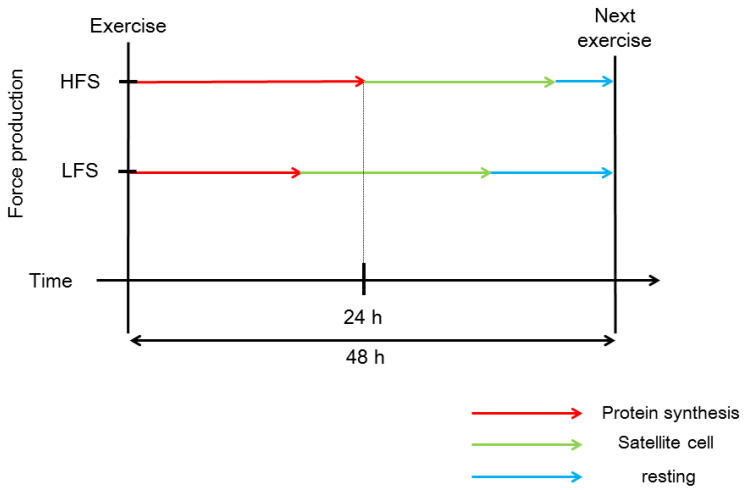

FIGURE 3-5. The time course changes between protein synthesis and involvement of satellite cell after HFS and LFS.

This illustration represents time course changes between protein synthesis and satellite cell. HFS: High-frequency (100Hz) electrical stimulation. LFS: Low-frequency (10Hz) electrical stimulation. The line shows protein synthesis phase (red line), involvement of satellite cell phase (green line), and resting phase (blue line).

Chapter 4

Not the high- but low-frequency intermitted electrical stimulation successively induce gastrocnemius muscle hypertrophy in Wistar rats

Introduction

Muscle contractile activity leads various morphological adaptations. Especially, skeletal muscle hypertrophy is well known as contraction induced response. This adaptation results increasing muscle mass, and cross sectional area (CSA). In humans and animals, resistance training and neuromuscular electrical stimulation (NMES) are often used to lead hypertrophy. Generally, resistance training and NMES for skeletal muscle hypertrophy are performed under high force output condition, such as over 70% of 1 repetition maximum in resistance training (4) and high frequency electrical stimulation in NMES (15, 16). However, it is thought that hypertrophic response is non-constant response. For example, in humans, Bamman et al. has classified into three groups, termed Non-responders (NR), Modest responders (MR), and Extreme responders (ER) according to hypertrophic rate after 16 weeks of progressively resistance training (91, 92). They reported that there were no significantly differences between NR, MR, and ER in their loading intensity and repetition during training period. Moreover, Kobayashi et al. also has shown that a response of training induced muscle hypertrophy is different according to rat strain i.e. Wistar and Sprague-Dawly (SD) (22). Thus, it is thought that muscle contraction induced hypertrophy is affected by genetic background and individual differences both in humans and animals.

Muscle mass changes are controlled by a balance between anabolic and catabolic reactions (73). The mammalian target of rapamycin complex1 (mTORC1) and its downstream target are thought as a critical regulator for muscle anabolism (36, 37). Notably, many studies have suggested that the

phosphorylation status of 70K-Da ribosomal protein kinase (p70S6K) is a well investigated molecule and used as an indicator for anabolic response (39, 74). On the other hands, muscle catabolism is also regulated by molecular response (78). Especially, the ubiquitin proteasome system is a major regulator for protein degradation molecules (93). Muscle RING finger 1(MuRF1) and muscle atrophy F-box (MAFbx) / Atrogin-1 is identified as a protein degradation biomarker (94, 95). These two molecules are highly expressed under atrophy condition.

Kobayashi et al. (22) examined whether expression of above molecules are different between SD and Wistar strains after twelve sessions of isometric training with HFS. They found that significant muscle hypertrophy was observed only in SD strain. They also found that p70S6K, which is anabolic indicator, was increased in SD strain. Moreover, MuRF1 and MAFbx/Atrogin-1 was decreased in SD rats. On the other hands, p70S6K phosphorylation, MuRF1 and MAFbx/Atrogin-1 were remained same after resistance training in Wistar strain. Thus, it is thought that skeletal muscle hypertrophy was not occurred by high frequency electrical stimulation in Wistar strain.

In chapter 2, I showed that intermittent low frequency electrical stimulation (LFS) induces skeletal muscle hypertrophy (20). In chapter 3, I suggested that LFS induced skeletal muscle hypertrophy is not implicated mTOR-dependent manner. Thus, I suppose that LFS can induce skeletal muscle hypertrophy in Wistar strain.

The purpose of present study was to investigate whether LFS induces skeletal muscle

hypertrophy. Additionally, I investigate protein expression both anabolic (phosphorylation level of p70S6K at residue of Threonine 389 site, which regulated by mTOR) and catabolic (MuRF-1 and MAFbx/Atrogin-1) indicator.

Materials and Methods

Animal care

Ten Wistar rats (9-10 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All animals were housed individually, in a 12-h light-dark cycle, with the lights kept on from 6:00 PM to 6:00 AM, and they were given food and water ad libitum. All procedures used in this study were approved by the ethical committee of the Nippon Sports Science University.

Experimental Design

Rats were divided evenly into a high-frequency electrical stimulation (HFS; 100 Hz) group and a low-frequency electrical stimulation (LFS; 10 Hz) group (n=5 each group).

Resistance Training Protocol

Under anesthesia, the right lower leg of each rat was shaved. The rats were then subjected to isometric training by electrical stimulation. Each rat was laid prone on a platform and its right knee was extended with a dynamometer, with the ankle joint positioned at an angle of 90°. The triceps of the right leg muscle was then stimulated (voltage: 30-35 V; pulse duration 4 ms: frequency: 100 Hz for HFS group and 10 Hz for LFS group) with a surface electrode (7.5 mm × 7.5 mm) that was connected to an electrical stimulator and isolator (Nihon Koden, Japan). The left medial

gastrocnemius muscle served as the untrained (UT) control.

For all training sessions, the triceps surae muscle was trained by stimulation for five 3s contractions, with a 5s interval between each contraction. Four sets in total were performed, with 3 min intervals between each set. All training sessions were conducted on every other day. The training and exercise schedule details are presented in Figure 1.

Animals were sacrificed 24 h after last training session. The medial gastrocnemius muscle was removed, weighed, and rapidly frozen in liquid nitrogen, and the right and left medial gastrocnemius muscles were triturated in liquid nitrogen and stored at – 80℃ until use.

Immunohistochemistry and Cross-sectional Area (CSA) measurement

To measure CSA, 10-μm-thick muscle cryosections were fixed with 2% paraformaldehyde and 0.25% picric acid in 0.1 M phosphate-buffered saline (PBS), for 15 min at room temperature. Following fixation, the sections were washed in 0.1 M PBS for 15 min and then postfixed in ice-cold methanol (-20℃) for 10 min. The sections were then washed 3 times in 0.1 M PBS for 5 min, and blocked in a 0.1 M PBS solution containing 10% goat serum and 1% Triton-X-100, for 1 h at room temperature. After blocking, the primary antibodies were applied (Laminin ;Sigma, USA) over night at 4℃. On the following day, the sections were washed in 0.1 M PBS, and the secondary antibody was applied overnight at 4℃. The sections were then washed twice in 0.1 M PBS for 10 min and

subsequently dried. Slides were viewed under a light microscope at x 10 magnification. The CSA quantification was performed by a computer application (NIH Image), with *n*=5 in each group. The CSA was counted to 100 fibers per sample (Total 2000 fiber are measured.).

Western Blotting Analysis

Frozen tissues were sonicated for 10 s in ice-cold RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 25mM Tris-Hcl pH 7.6, 150mM NaCl, 5mM EDTA, 1% NP-40, 1% sodium deoxycholate, and protease and phosphatase inhibitor (Thermo scientific). Homogenate were then centrifuged 15,000×*g* for 20 min at 4℃ and supernatant collected for analysis. Sample protein concentrations were determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts (10μg) of protein from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on TGX gels (Bio-Rad Laboratories, Hercules, CA, USA). After end of gel running, separate proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat dry milk in TBS-T (Tris-bufferd saline mixed with 0.1% Tween 20) for 60 min followed by an overnight incubation at 4℃ with dilutions of each primary antibodies dissolved in TBS-T containing 5% non-fat dry milk (p70S6K, MAFbx/Atrogin-1, and MuRF1) or 5% BSA (α-tubulin). Membranes were washed for 15 min in TBS-T and then incubated for 1 h at room temperature in 5% non-fat dry

milk TBS-T containing horseradish peroxidase conjugated secondary antibodies. After 15 min of washing in TBS-T, the blots were detected using chemiluminescence reagent (West femto maximum, Thermo scientific). Protein expression was measured by computer application (CS analyzer, ATTO). Phosphorylation of p70S6K at T389 antibody, MAFbx/Atrogin-1, and MuRF1 primary antibody purchased from Santa Cruz Biotechnology. The primary antibody of α-tubulin was purchased from Cell signaling technology. Secondary HRP-conjugated antibodies purchased from Santa Cruz Biotechnology and Cell signaling technology.

Statistical Analysis

The statistical differences between the trained (T) and untrained (UT) legs were determined by paired *t*-tests. Concurrently, the statistical differences between HFS and LFS treatments were determined by unpaired *t*-tests. All numbers are expressed as the mean ± SD. $P < 0.05$ was considered to denote acceptable significance.

Results

Body weight and Muscle weight after 6 sessions of training.

Table 4-1 shows body weight and muscle weight in Wistar rats after six sessions of isometric training. HFS was no significant difference T from UT. On the other hands, LFS T was significantly higher than UT ($p<0.01$). However, there are no significant differences between LFS T and HFS T.

Cross sectional area measurement

Figure 4-1 represents the histogram of CSA of each group. Figure 4-1A displays that the CSA of HFS T was abound more than HFS UT at 600-1200μm^2. However, the CSA of HFS T was lessened more than HFS UT at over 1200μm^2. Figure 4-1B shows that the CSA of LFS T was abound more than its UT at 800-1400μm^2.

Table 4-2 shows the average value of CSA in each group. There were no significantly differences between each T and UT and between HFS UT and LFS UT. However, there was significance between HFS T and LFS T ($p<0.05$).

Expression of phosphorylated p70S6K

Figure 4-2 shows that phosphorylation status of p70S6K at the residue of T389 site. HFS T was significantly higher than its UT ($p<0.05$). Conversely, LFS T was no significance from LFS UT.

Protein expression of MAFbx/Atrogin-1 and MuRF-1

Figure 4-3 represents the expression of MAFbx/Atrogin-1 (3A) and MuRF-1 (3B) after 6 sessions of isometric training. In Fig 4-3A, MAFbx/Atrogin-1 in HFS T was higher expression than HFS UT ($p<0.05$). Conversely, LFS T was no difference from its UT. Moreover, the expression of MAFbx/Atrogin-1 in HFS was higher than LFS ($p<0.05$). Fig 4-3B shows that the expression of MuRF-1 in HFS T was higher than its UT ($p<0.05$). On the other hands, no significance between LFS T and LFS T. However, there was no significance between HFS and LFS.

Discussion

In this study, I examined whether LFS induces skeletal muscle hypertrophy in Wistar strain, which is insensitive to high mechanical stimulation to lead skeletal muscle hypertrophy (22). I found that LFS increased skeletal muscle mass in Wistar strain whereas significantly hypertrophy did not occur with HFS. In this section, I try to discuss that reason why muscle hypertrophy was induced by LFS in Wistar strain.

A key finding is that LFS can induce significantly mass change in Wistar strain. Although HFS did not occur significantly mass change (3.8 ± 4.1 %, from HFS UT), LFS was succeeded (6.6 ± 3.2 %, from LFS UT; $p<0.05$). To confirm this change, I performed the measurement of CSA and found that the average value of CSA in LFS T was slightly increased from LFS UT (see Table 4-2.). Therefore, I can be confirmed that LFS can induce skeletal muscle hypertrophy in Wistar strain.

Previously, Kobayashi et al. (22) have reported that Wistar strain did not induced mass change after twelve sessions of isometric training with high frequency electrical stimulation. In present study, I investigated the CSA was not significantly increased in both HFS and LFS from its UTs. Especially, despite significantly increasing muscle mass, the CSA was few changed from LFS UT. I performed six sessions (13days) of isometric training in this study. It might be thought that this condition is short period as training session for increasing CSA level. Conceivably, it might be thought that if LFS training is performed longer than six sessions, skeletal muscle hypertrophy

becomes pronounced in fiber level.

Electrical stimulation with high-frequency is generally used for skeletal muscle hypertrophy It is usually thought that skeletal muscle hypertrophy engages type II fiber recruitment. Accordingly size principle theory (96), muscle recruitment is gradually shifted type I fibers to type II fibers along with increasing force production under conscious resistance training. Therefore, skeletal muscle hypertrophy is preferentially occurred in type II fibers respond to external stimuli (48). In present study, I used NMES model, which is used electrical stimulation for muscle mobilization. Previous studies have shown that muscle recruitment by electrical stimulation is random (19, 20). Additionally, medial gastrocnemius muscle is type II fibers-dominant muscle (20, 22, 97). Therefore, it is thought that type II fibers were mainly used for muscle contraction in this study. Although type II fibers recruited by muscle contraction, hypertrophic response may not be occur. In fact, several studies have shown that hypertrophic response by progressively high intensity resistance training is non-constant (91, 92, 98). This disparity may be attributable to genetic and individual background.

To date, skeletal muscle hypertrophy regulated by intracellular signaling (36, 73). Especially, mTOR and its downstream target, p70S6K are key factor for protein synthesis and translational control (36, 37). The phosphorylation of p70S6K indicates the activation of mTOR. Although HFS has shown that phosphorylation of p70S6K was significantly increased after six sessions of isometric training, HFS cannot be succeeded significantly mass change in this study. Conversely, despite a

lack of increasing cellular response, LFS was significantly increased muscle mass in Wistar strain. In chapter III, I showed that LFS-induced muscle protein synthesis might be independent on mTOR. Thus, LFS-induced skeletal muscle hypertrophy was occurred in Wistar strain. As one of reason for failure hypertrophy in HFS, Wistar strain might be genetically has a down regulator of mTOR.

Kobayashi et al. (22) has reported that intracellular signaling regulated muscle anabolism in Wistar strain is suppressed by its catabolism. In fact, I investigated MuRF1 and MAFbx/Atrogin-1, which are catabolic marker and are also significantly increased in HFS. Thus, catabolic response suppresses anabolic response in HFS. As other pathway for skeletal muscle hypertrophy, Myostatin, which is a transforming growth factor β (TGF-β) family member, is well-known as one of negative regulator in skeletal muscle (99, 100). Myostatin can phosphorylate its downstream target such as Smad proteins (Smad 2/3) via activin I and II receptors and suppress muscle anabolism (99). TGF-β network have other pathway, named Bone Morphogenetic Protein (BMP) pathway (89, 101). In contrast to Myostatin-Smad axis, BMP signaling regulate muscle mass as positive regulator (101). Possibly, these two pathways regulate LFS-induced skeletal muscle hypertrophy. Therefore, further analysis in LFS-induced skeletal muscle hypertrophy at genetic and molecular level was needed.

In summary, the examination in this study demonstrates that LFS can induce skeletal muscle hypertrophy in Wistar strain. Moreover, it has possibility that LFS suppresses expression of catabolic-related protein. Thus, it might be thought that LFS can induce skeletal muscle hypertrophy

without affecting genetic background and individual difference.

Table 4-1. Body weight and Muscle weight after 6 sessions of training

		HFS	LFS
Body weight (g)		340.75±26.63	351.26±10.77
Medial Gastronemius muscle (mg)	UT	784.16±43.21	787.86±35.66
	T	813.16±28.92	839.06±34.67**
Medial Gastronemius muscle/ Body weight (mg/g)	UT	2.31±0.12	2.24±0.06
	T	2.40±0.17	2.39±0.06**
% change (T/UT)		3.8±4.1	6.6±3.2

HFS: high frequency electrical stimulation, LFS: low frequency electrical stimulation, T: trained leg , and UT: untrained leg. Values are mean ±SD. ** $p<0.01$ vs. UT.

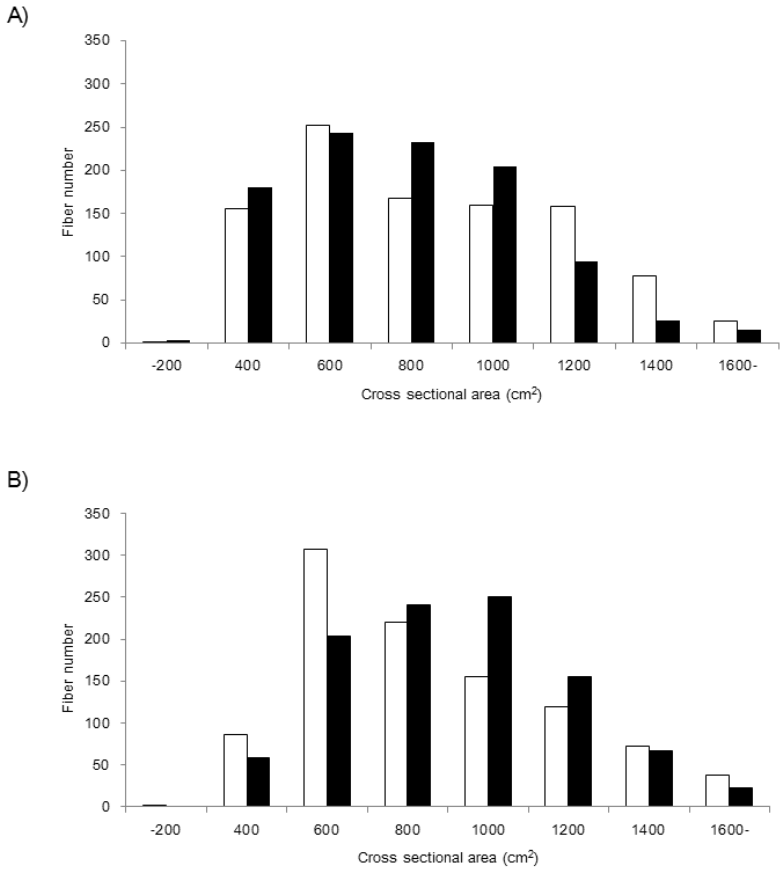

FIGURE 4-1. CSA measurement. (A) The CSA after 6 sessions of training in HFS. (B) The CSA after 6 sessions of training in LFS. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. White bar expressed untrained leg in each group. Black bar expressed trained leg in each group.

Table 4-2. The average values of CSA

	HFS		LFS	
	UT	T	UT	T
Cross sectional area (cm^2)	749.64±131.97	683.97±37.94	757.03±122.21	806.82±82.99 *

HFS: high frequency electrical stimulation, LFS: low frequency electrical stimulation, T: trained leg , and UT: untrained leg. Values are mean ±SD. * $p<0.05$ vs. HFS T.

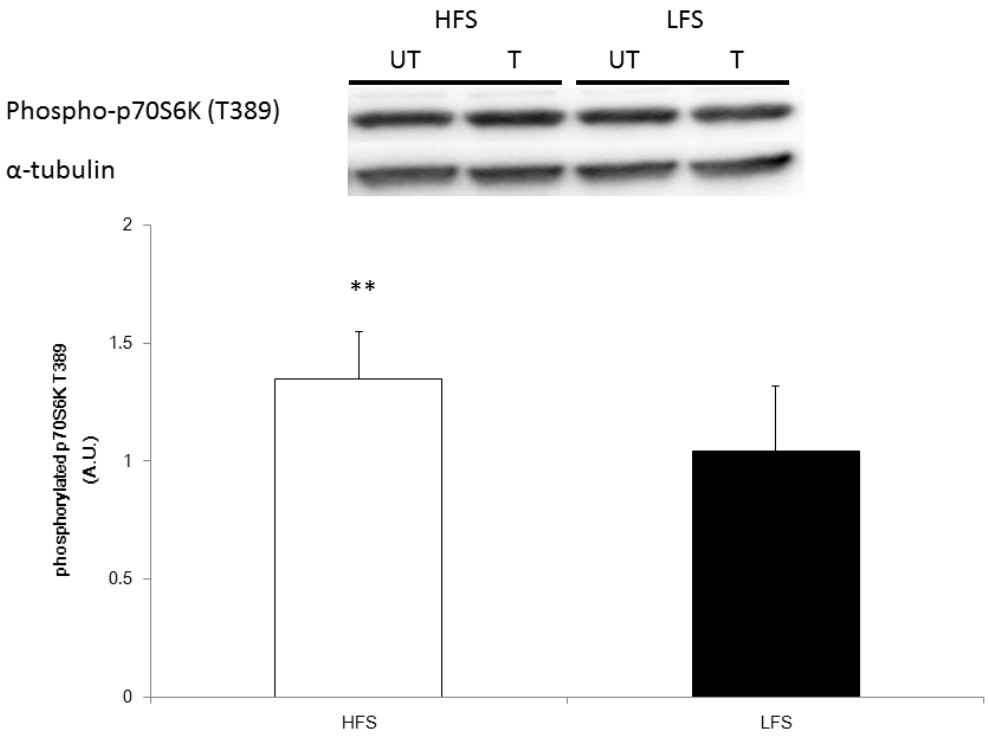

FIGURE 4-2. Expression of phosphorylated p70S6K. The level of p70S6K phosphorylation at Thr389. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. The relative value was defined as the ratio of trained leg against untrained control leg. All values are the mean ± SD. $^{**}p < 0.01$ and UT.

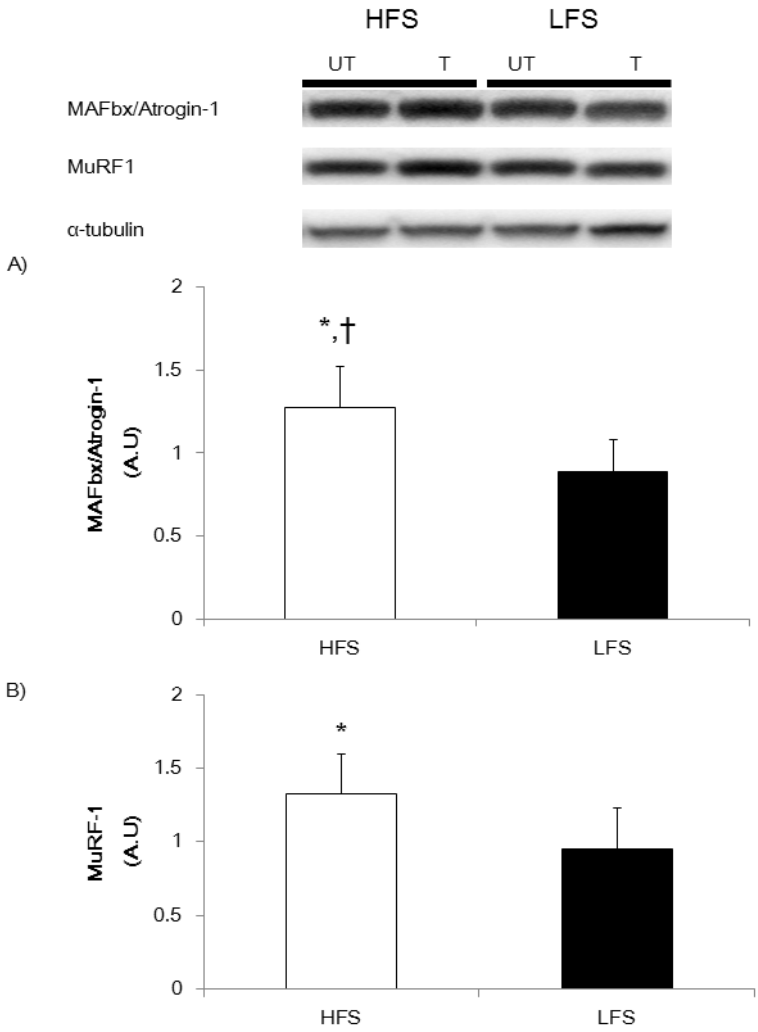

FIGURE 4-2. Protein expression of MAFbx/Atrogin-1 and MuRF-1. (A)The expression of MAFbx/Atrogin-1. (B)The expression of MuRF-1. HFS: high-frequency electrical stimulation group. LFS: low-frequency electrical stimulation group. The relative value was defined as the ratio of trained leg against untrained control leg. All values are the mean ± SD. $^{*}p < 0.05$vs. UT; †p<0.05 vs. LFS.

Chapter 5

Summary and Future perspective

The key findings of this thesis are following:

1) I succeeded to develop that intermittent low-frequency electrical stimulation induces skeletal muscle hypertrophy on rat gastrocnemius muscle (chapter2).

2) The molecular mechanisms of intermittent low-frequency electrical stimulation–induced skeletal muscle hypertrophy at least through p70S6K. However, the detail of its mechanism is still unclear (chapter3).

3) Intermittent low frequency electrical stimulation also induces skeletal muscle hypertrophy in Wistar rats, which is insensitive to lead hypertrophic response (chapter4).

To date, although high intensity resistance training is most helpful methods, several low intensity training protocol have also reported to lead skeletal muscle hypertrophy. On the other hand, it has thought that NMES also helpful method for skeletal muscle hypertrophy (15). Basically, NMES is often used for rehabilitative application while NMES has also applied for healthy subjects. In fact, Gondin and coworkers (16) have shown that NMES with high frequency (75 Hz) and isometric contraction training increases muscular strength and mass size in healthy humans. Concurrently, our previously reports (20, 22, 23) have also shown that high frequency electrical stimulation (HFS; 60-100 Hz) with isometric contraction induces skeletal muscle hypertrophy in animals. On the other hand, low frequency electrical stimulation is also applied as endurance-like training (15, 27). This

NMES model expresses phenotypic alteration in muscle fiber and maintaining of mass size (30).

In chapter 2, I suggested that intermittent NMES with low-frequency (LFS) electrical stimulation induces skeletal muscle hypertrophy. LFS-induced skeletal muscle hypertrophy has several characters such as low force generation comparison with HFS, did not change fiber type, and the possibility of mTOR-independent hypertrophy. This is novel model to lead muscle hypertrophy and these characteristics are interesting in terms of physiology. Especially, hypertrophic response is independent on mTOR signaling model is very curious.

In chapter 3, I examined to confirm whether LFS-induced muscle protein synthesis (MPS) through mTOR signaling. As a result, I identified that LFS-induced MPS is at least through p70S6K. Numerous studies have investigated that activation of mTOR is measured by its downstream substrate phosphorylation such as p70S6K and eukaryotic initiation factor 4E-binding protein (4EBP1) (35, 73). In this study, although phosphorylation of mTOR by LFS is decreased, it is unclear that phosphorylation of mTOR is suggested its activation (88). However, mTOR is not alone involved in protein synthesis. Recent studies have reported that several signaling pathway or molecules associated with muscle mass control (89, 101). It has possibilities that the discovery of molecular mechanism of LFS-induced MPS leads to novel molecule associated with muscle hypertrophy.

High frequency electrical stimulation has a potential for improving muscular strength. However,

Bamman and co-workers (91, 92, 98) have reported that high intensity resistance training is cannot constantly induce hypertrophic response. Accordingly their work, the identical load intensity is resulted different hypertrophic response and they classified into three groups based on hypertrophic rate, named Non-responder (NR), Moderate responder (MR), and Extreme responder (ER). In animal experiment, Kobayashi et al. (22) have also suggested that hypertrophic response is different depend on rat strain. They have reported that high frequency electrical stimulation did not succeed skeletal muscle hypertrophy in Wistar rat. Taken together, these are suggested that hypertrophic response in skeletal muscle depend on genetic or individual background. In Chapter 4, I applied LFS and can succeed skeletal muscle hypertrophy in Wistar rat. It is thought that this evidence is important for applying to humans. Therefore, I feel necessity to perform this LFS model in human.

In conclusion, I thought that my study model, named LFS, serve as the foundation of future development of various scientific views.

Chapter 6

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Chapter 7

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Chapter 8

List of publications

Original Article

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