Effect of chronic muscle contraction on expression of contractile and metabolic proteins in mouse primary cultured myotubes

Yoshitaka Mita[#], Miyuki Ito[#], Mio Yamada, Nobuharu L. Fujii,
Yasuko Manabe^{*} and Yasuro Furuichi^{*}

*Department of Health Promotion Sciences, Graduate School of Human Health Sciences, Tokyo Metropolitan University,
1-1 Minami-Osawa, Hachioji, Tokyo 192-0397, Japan*

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Abstract Endurance exercise induces skeletal muscle adaptations such as fiber-type switching, mitochondrial biogenesis, angiogenesis, and the enhancement of glucose disposal, all of which ameliorate metabolic dysfunction. Since many factors such as body temperature, pH, osmolality, the secretion patterns of neurotransmitters, and humoral factors, change during exercise, it is not easy to determine precisely how each factor contributes to exercise-induced adaptations. To determine these contributions, there is need for experimental studies using an in vitro muscle culture system focusing on a single added stimulus. In this study, we focused on whether contractile stimulation is itself responsible for inducing skeletal muscle adaptations. We constructed a chronic contraction model in mouse primary myotubes and investigated which type of contractile stimulation could induce muscle fiber switching and/or metabolic adaptations. We tested five sets of contractile stimulus conditions, including tetanus and twitch, for different stimulation periods. Of these, when myotubes were stimulated by 6 V/15 mA electric pulses at 1 Hz (20 ms contraction followed by 980 ms relaxation) for 24 hours, we observed a significant increase in the expression of myosin heavy chain (MyHC) I protein, a marker protein for type I (oxidative) myofiber, and a tendency for MyHC IIa expression to increase, a marker protein for type IIa fiber (the most oxidative myofiber out of the type II isoforms). However, the same conditions did not induce any change in the expression of GLUT4, COX IV, and hexokinase II, proteins related to the transport of glucose and metabolism. These results suggest chronic contractile stimulation does not induce the expression of proteins related to metabolism, but it does regulate the expression patterns of MyHC. This chronic contraction model has the potential to clarify the molecular mechanisms underlying the induction of oxidative myofibers in response to muscle contraction.

Keywords : skeletal muscle, contractile myotube, electric pulse stimulation, primary cultured cell

Introduction

Endurance exercise induces skeletal muscle adaptations that ameliorate metabolic dysfunction. But given the number of physiological factors that change during exercise (e.g., body temperature, pH, osmolality, neurotransmitter secretion patterns, and humoral factors), it is a challenge to quantify the exact contribution of each factor to exercise-induced adaptations. The role of muscle contraction itself as a single factor is a particular focus of research¹⁾.

Chronic exercise training induces a transformation of muscle fiber type from glycolytic to oxidative, concomitant with functional adaptations including mitochondrial

biogenesis, angiogenesis, and an enhancement in glucose disposal²⁻⁵⁾. Recent studies have drawn particular attention to the detailed mechanisms underlying these adaptations, since muscle metabolic adaptations are closely related to whole-body metabolic response, and thus can ameliorate metabolic dysfunctions such as insulin resistance⁶⁾.

Myofibers are broadly classified as type I (oxidative) and type II (glycolytic) based on their contractile and metabolic properties. Each fiber type has a specific subtype of myosin heavy chain (MyHC) isoform. Fibers expressing MyHCI are called type I fibers and show oxidative properties; fibers expressing MyHCII are called type II fibers and show glycolytic properties. Type II fibers are subdivided into type IIa, IIx, and IIb, which are preferen-

[#]Co-first authors

^{*}Co-corresponding authors: ymanabe@tmu.ac.jp; furuichi@tmu.ac.jp

Abbreviations: COX: cytochrome c oxidase subunit; GLUT4: glucose transporter member 4; MyHC: myosin heavy chain

tially expressed as MyHC IIa, IIx, and IIb, respectively. Notably, type IIa fiber is defined as oxidative even though it is type II, because it exhibits contractile characteristics which are intermediate between type I and type IIx/IIb fibers⁷⁾. Skeletal muscle tissues consist of a mixture of these fiber types, and their properties depend on the ratio in which each fiber type is present. Although the body's fiber type composition is recognized as genetically determined, exercise training also has the potential to induce fiber-type switching⁸⁾.

Forced muscle contraction induced by electrostimulation as a model of contractile stimulation has shown that contractile stimulation itself is likely to be one of the primary factors that changes muscle-fiber characteristics in cats⁹⁾ and rats¹⁰⁾. In agreement with this idea, the activation patterns of the transcription factor NFAT (nuclear factor of activated T cells), a key transcriptional factor which regulates MyHC expression patterns, were restricted by the stimulation of motor neurons with an electric pulse at a specific frequency¹¹⁾. Contractile stimulation also increased the promoter activity of PGC-1 α (peroxisome proliferator-activated receptor- γ coactivator-1 α)¹²⁾, known to be a key regulator of muscle adaptive processes. These findings suggested that contractile stimulation is a critical factor for the induction of muscle adaptations. However, because an *in vivo* approach was used in these previous studies involving multiple factors derived from the whole body, it remains unclear whether mechanical contractile stimulation is the only factor involved in the induction of these adaptations, or whether other concomitant changes such as neurotransmitter secretion patterns or humoral factors may also contribute.

We have previously developed acute myotube contraction models using C2C12 cells and skeletal muscle stem cells (satellite cells)^{13,14)}. These cell culture-based models are suitable to investigate whether contractile stimulation is a sufficient factor to induce muscle adaptations in the absence of nerve and other blood-derived factors. We conducted the present study to determine whether physical contractile stimulation alone is sufficient to induce fiber-type switching and/or metabolic adaptations similar to those induced by *in vivo* exercise training.

Materials and Methods

Animals. Adult (7- to 15-week-old) male C57BL/6 mice were obtained from Sankyo Lab (Tokyo), housed at 23°–25°C with a 12-hr light/dark cycle, and fed a normal chow diet (Oriental Yeast, Tokyo) and water *ad libitum*. The care of the mice was in accordance with the guidelines of the Experimental Animal Use Committee of Tokyo Metropolitan University and followed the guidelines for the proper conduct of animal experiments established by the Science Council of Japan (A31-19).

Primary satellite cell isolation and culture. Mice were sacrificed by cervical dislocation. Extensor digitorum longus (EDL) muscles were isolated and digested in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.2% (w/v) Type I collagenase (260 U/mg) and 1% (v/v) penicillin-streptomycin, as described previously¹⁴⁾. Satellite cells were cultured in growth medium, i.e., no glucose DMEM supplemented with 30% (v/v) fetal bovine serum (FBS), 1% (v/v) GlutaMAX™, 1% (v/v) chicken embryo extract, 10 ng/mL basic fibroblast growth factor (bFGF) and 1% (v/v) penicillin-streptomycin at 37°C with 5% CO₂. After 6 days of culture, myoblasts derived from satellite cells were reseeded into eight-well rectangular plates coated with Matrigel (Corning, NY, USA) at a density of 4.5×10⁵ cells/well with 2 mL of growth medium. One day after the seeding, the medium was switched to differentiation medium, i.e., high glucose DMEM GlutaMAX supplemented with 5% (v/v) horse serum and 1% (v/v) penicillin-streptomycin.

Myotube contraction. Four days after differentiation, the eight-well plates were connected to an electrical stimulation apparatus, i.e., an eight-well C-Dish (Ion Optix, Milton, MA), and the myotubes were stimulated by an electric pulse generator (Nihon Kohden, Tokyo), as described previously¹³⁾. The differentiated myotubes were stimulated with electric pulses under several sets of conditions (Table 1) followed by harvesting of the myotubes immediately after the sessions. Myotubes without electric pulses were used as the basal condition (Basal). The con-

Table 1. Electric pulse conditions

Condition	#1	#2	#3	#4	#5
Voltage (V)	8	8	8	8	6
Current (mA)	5	5	5	5	15
Frequency (Hz)	100	10	100	100	1
Duration (msec)	3000	5	0.1	0.1	20
Session (hrs/day)	4.5	1.5	24	24	24
Number of days	-	12	2	4	1

tractile activity of the cells was evaluated using a motion analyzer (VW-9000; Keyence, Osaka, Japan). After contraction, conditioned medium was collected for a Lactate Dehydrogenase (LDH) assay, and cells were harvested with 200 μ L of ice-cold lysis buffer as described previously¹³. Harvested cells were sonicated and centrifuged at 13,000 g for 15 min at 4°C. The supernatant was used as the cell lysate. The protein concentration of the cell lysate was determined by the Bradford method.

Immunoblotting. Cell lysates were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with Tris-buffered saline containing 0.1% Tween 20, either 5% bovine serum albumin, 5% non-fat dry milk, or 2.5% non-fat dry milk and 2.5% bovine serum albumin. The membranes were incubated overnight with the appropriate primary antibodies of anti-MyHC I, anti-MyHC II (Sigma-Aldrich, St. Louis, MO), anti-MyHC IIa (sc-71; Developmental Studies Hybridoma Bank; DSHB), anti-MyHC IIb (BF-F3; DSHB), anti-myoglobin (Abcam, Cambridge, UK), anti-GLUT4, anti-COX IV, anti-hexokinase II, anti-GAPDH, anti-mTOR (7C10), anti-phospho-mTOR (Ser2448) (D9C2), anti-p70 S6 kinase (49D7), or anti-phospho-p70 S6 kinase (Thr389) (Cell Signaling Technology, Danvers, MA). Secondary antibodies of anti-mouse IgG conjugated to horseradish peroxidase (GE Healthcare, Buckinghamshire, UK), and anti-mouse IgM μ chain conjugated to horseradish peroxidase (Abcam, Cambridge, UK) were used for detection with enhanced chemiluminescence (PerkinElmer, Waltham, MA).

LDH assay. LDH activity in the conditioned medium was measured using an LDH assay kit following the manufacturer's instructions (Roche, Basel, Switzerland).

Statistical analyses. All values are shown as the mean $\pm$ standard error of the mean (SEM). Student's t-test was used to compare pairs of groups. Probability (p)-values <0.05 were considered significant.

Results

Culturing mouse primary myotubes under chronic contraction. Five sets of contraction conditions were as in vitro training models; these are provided in Table 1. Under conditions #1 and #2, the myotubes detached from the culture dish before the contraction sets were completed, most likely due to overload of the cells (Supplemental Fig. 1A, B). Conditions #3 to #5 had shorter durations, lower frequencies, and/or less stimulation time per day to prevent the damage of myotubes. The resulting myotubes were observed to be significantly contracted by the end of the contraction period without any obvious damage, as evaluated visually (Supplemental Fig. 1A, C). These con-

tracted myotubes were also observed to show rhythmic contractions synchronized to the electric pulses (Supplemental movie 1, 2).

Effects of chronic contraction on protein expression. We next determined the protein expression levels of MyHC I and MyHC II as markers of fiber type, and GLUT4, hexokinase II, myoglobin, and COX IV as markers of exercise training-induced adaptations¹⁵⁻¹⁷. In terms of contraction modalities, conditions #3 and #4 were set as tetanus, while condition #5 was set as twitch. We show the typical results obtained from repeated experiments (Fig. 1A-D). The expression level of MyHC I was significantly increased after 24 hours of contractions under condition #5 (Fig. 1C); the MyHC IIa expression level also tended to increase ($p = 0.07$). On the other hand, MyHC IIb, fast type MyHC, and other proteins related to metabolism showed no change in expression levels (Fig. 1C, D). To assess the extent of cell damage due to the electric pulses or muscle contractions, LDH concentration in the conditioned medium was measured. LDH is present in the cytoplasm of intact cells and is rapidly released into the culture medium after cell damage. LDH activity in the conditioned medium was not increased by myotube contraction under condition #5, indicating that the myotubes were not injured (Supplemental Fig. 2A). None of the other contraction conditions changed the expression level of any protein quantified here.

Discussion

We established a chronic muscle contraction model which specifically upregulates the expression of MyHC I, and tends to increase MyHC IIa expression in mouse primary cultured myotubes. It should be noted that previous studies have also reported the induction of MyHC I expression in cultured myotubes by the application of low frequency chronic electrical stimuli¹⁸⁻²¹. However, these results do not report the expression of MyHC IIa. This is also the first time that conditions have been identified which, when applied over a full 24-hour period, cause upregulation of MyHC I expression in mouse primary cultured myotubes. The conditions established in this study offer a tool to elucidate the mechanism behind the expression of MyHC in response to muscle contraction. In addition, metabolic proteins, which are also known to be induced by endurance exercise, were not increased after chronic contraction. These results thus suggest that mechanical contractile stimulation is sufficient to regulate MyHC expression, but not adequate to change the expressions of metabolic genes.

We observed that contractions under condition #5 upregulated the expression of MyHC I (Fig. 1C), but with no additional induction of phospho-mTOR and p70 S6 kinase, surrogates of protein synthesis, (Supplemental Fig. 3A). Moreover, the amount of protein in lysates of

Fig. 1 Protein expression of MyHC isoforms, hexokinase II, GLUT4, myoglobin, and COX IV with or without chronic contractions. Myotubes were stimulated with an electric pulse as summarized in Table 1. Myotubes were immediately harvested after contraction sessions. Representative blots and densitometric analysis results for (A) condition #3 ($n = 3-4$), (B) condition #4 ($n = 4$) and, (C, D) condition #5 (C: $n = 5-8$, D: $n = 8$) are shown. Data are given as mean $\pm$ SEM. Differences from the basal condition are considered significant when $p < 0.05$.

myotubes with and without electrical stimulation, quantified with the Bradford method, were not significantly different (Supplemental Fig. 3B). These results at least suggest that condition #5 did not activate protein synthesis in only a specific myotube. Previous reports showed that primary myotubes co-express both MyHC I and MyHC II isoforms^{22,23}. In view of these observations, the increase in MyHC I expression under condition #5 are probably due to the upregulation of MyHC I expression in a MyHC hybrid myotube in response to the contractions. However, we note that there is a need to apply single-cell analysis of myotubes for analysis of changes in MyHC expression in each myotube in the future. It is also known that a low frequency electrical impulse pattern induces localization of NFATc1 into the nuclei of the myotubes, which promotes the expression of MyHC I mRNA¹⁸. Conditions #3 and #4 were likely to be higher frequency than the impulse pattern which induces the expression of MyHC I. On the other hand, condition #5 was set to a lower frequency than all the other conditions in this study, and assumed to be low enough to induce MyHC I expression. One can consider this in the context of how differences in exercise modalities, such as resistance training or aerobic training, induce different responses in skeletal muscle. For example, resistance exercise preferentially induces hypertrophy of type II muscle fibers²⁴. It is therefore possible that increasing the duration and frequency of contraction conditions, mimicking what might be seen during resistance exercise, may elicit upregulation of the

expression of MyHC II. However, it is difficult to increase the intensity of the contraction conditions adopted in the present cell model because monolayers of primary cultured myotubes are susceptible to damage. To overcome this limitation, we need to find new strategies to prevent the collapse of cells. It will also be necessary to generate high-force stimulation like in three-dimensional culture systems²⁵.

Chronic exercise has been demonstrated to activate intercellular signaling pathways that increase the expression of genes involved in the slow-twitch contractile apparatus and metabolic genes^{2,26,27}. Since expression of MyHC I and MyHC IIa, which are known as slow type MyHC, were induced by condition #5, it can be said that this condition was able to reproduce one of the responses seen during chronic training adaptations in vivo in skeletal muscle. However, our present data indicate that the continuous contraction did not induce metabolic genes. It is thus likely that a consequence of physical contraction in skeletal muscle cells is the regulation of MyHC determination, but not metabolic genes. In fact, the results of previous studies have suggested that the MyHC determination pathway and metabolic pathway are parallel^{28,29}. In order to upregulate the expressions of genes related to oxidative metabolic properties, skeletal muscle cells may need to be stimulated by other factors involving non-muscle cells, such as upregulating glucose metabolism or mitochondrial respiration^{30,31}.

Muscle atrophy occurs under a variety of conditions. In

particular, muscle disuse involving loss of motor neurons and mechanical loading causes the atrophy of type I myofibers³²⁾. Although efforts have been made to prevent this atrophy, exercise programs and nutritional interventions were unable to maintain type I myofiber size and peak force during 60 days of bed rest³³⁾. Since we demonstrated that chronic contraction evoked by electrical stimulation induces the expression of MyHCI, a treatment using electric pulse stimulation may be effective for patients' recovery from atrophy of type I myofibers.

In summary, the expression of MyHCI and MyHC IIA protein could be induced by 24-hour chronic contraction in mouse primary myotubes, suggesting that "chronic contraction" is a key to regulating the expression of slow type MyHC. Although other well-known metabolic proteins enhanced by endurance training were not upregulated under this condition, this stimulus tool provides a new technique for exploring the detailed mechanisms underlying the induction of oxidative myofibers in response to muscle contraction.

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Conflict of Interests

The authors declare no conflict of interests regarding this study.

Author Contributions

YMi, MI and MY performed and analyzed the experiments. YMi, NLF, YF, and YMa designed the experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Supporting Information

Supplemental figures and movies for this article are available online.

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