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Continuous Therapeutic Ultrasound Inhibits Progression of Disuse Atrophy in Rat Gastrocnemius Muscles

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Abstract. [Purpose] This study examined the effects of therapeutic ultrasound on the inhibition of disuse atrophy progression in rat gastrocnemius muscles. [Subjects and Methods] Male Wistar rats were randomly divided into control, ultrasound (US) and sham treatment groups. In the US and sham group, bilateral ankle joints of each rat were fixed in full plantar flexion with a plaster cast over a 2-week period. Continuous ultrasonic energy (frequency, 1 MHz; intensity, 1.0 W/cm^2) was delivered to the gastrocnemius muscle of the US group over a 2-week immobilization period. [Results] In the US and sham groups, type IIa and IIb mean muscle fiber diameters decreased significantly relative to the control group. In the US group, muscle fiber diameters of all fiber types were markedly larger than those of the sham group. Heat shock protein 70 (HSP70) content of the US group increased significantly in comparison with the sham group. The levels of basic fibroblast growth factor were substantially higher in the US group relative to the control and sham groups. However, meaningful differences in insulin-like growth factor I level were not evident between the groups. [Conclusion] Continuous therapeutic ultrasound inhibits the progression of disuse muscle atrophy via elevation of HSP70 levels in skeletal muscle.
Key words: Ultrasound, Muscle atrophy, Heat shock protein 70

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INTRODUCTION

Previous studies involving animals and humans have examined changes in skeletal muscle temperature during continuous mode ultrasound irradiation. Locke and Nussbaum[1)] reported that continuous ultrasonic wave (frequency, 1 MHz; intensity, 1.0 W/cm^2) application to the triceps surae muscles of rats for 15 min elevated muscle temperature by 2 to 6 °C. In our earlier investigation[2)], ultrasound irradiation under conditions identical to those of the foregoing study led to an increase of 6 °C in the triceps surae muscle temperature of rat. After 10 min continuous ultrasound (frequency, 1 MHz; intensity, 1.5 W/cm2) application to the medial gastrocnemius muscle for 10 min in humans, the mean temperature reached 40.3 °C, an increase of 4.9 °C [3)]. Thus, ultrasound is an appropriate therapeutic modality for warming deep tissue, e.g., skeletal muscle. Following soft tissue injury, from the subacute to the chronic phase, clinicians often employ ultrasound in order to harness its thermal effect[4)].

At the cellular level, increases of 3 to 5 °C above baseline temperatures in muscle have been shown to induce the expressionof heat shock proteins (HSP)[5)]. HSP 70, one of several HSP families, is induced in the cytoplasm by warming and is thought to function in the promotion of protein synthesis, the repair of denatured proteins and the transport of denatured proteins in which lysosomal degradation cannot be repaired[6)]. HSP 70 also plays an important role in chaperoning nascent peptides during translation, cellular protein transport and stability. Ku and Thomason[7)] reported that the decline in HSP70 in myocytes is a potential mechanism for the decreased translation observed during disuse muscle atrophy. If the warming of skeletal muscle by continuous mode therapeutic ultrasound can induce HSP70 expression in muscle, disuse muscle atrophy may be preventable. However, after single continuous mode ultrasound application to the lower leg muscles of rats, HSP 70 levels of the soleus, plantaris and gastrocnemius muscles failed to increase[1)]. In contrast, following the delivery of continuous mode ultrasound on four consecutive days to the lower leg muscles of rats, elevated HSP 70 levels were observed in the white gastrocnemius muscle[8)]. Therefore we hypothesized that HSP 70, induced by ultrasonic heating, would inhibit the progression of disuse atrophy in skeletal

muscle.

On the other hand, pulsed therapeutic ultrasound induces non-thermal effects which cause biological reactions and the expression of growth factors is a feature of these reactions. Reher et al.[9] noted that pulsed ultrasound irradiation (frequency, 1 MHz; pulse, 1:4; intensity, 0.1 W/cm^2) of osteoblasts leads to the expression of basic fibroblast growth factor (bFGF). Naruse et al.[10] documented the upregulation of insulin-like growth factors (IGF) mRNA in ST2 cells at 24 h following exposure (20 min) to low-intensity pulsed ultrasound. FGF functions as an activator and as a mediator of skeletal muscle hypertrophy[11]. IGF-I plays a major role in skeletal muscle (re)growth in an autocrine / paracrine fashion[12]. Rommel et al.[13] demonstrated the importance of the Akt (protein kinase B) pathway, which is downstream of IGF-I, muscle (re)growth following atrophy. The expression of these growth factors in muscle in response to ultrasound irradiation might help to prevent disuse muscle atrophy. However, the biophysical effects of ultrasound irradiation in skeletal muscle remain unclear. Accordingly, the objective of this study was to determine whether continuous therapeutic ultrasound inhibits the progression of disuse muscle atrophy in the rat gastrocnemius muscle.

METHODS

All experiments and procedures were approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University.

Fifteen 8-week-old male SPF Wistar rats were obtained from Kyudo Co., LTD (Tosu, Saga, Japan). The animals were housed in cages inside a room with a 12-h dark/light cycle. The temperature and relative humidity of the room were maintained at 25 °C and 50%, respectively. Rats were able to move freely in cage using their forelimbs. Food and water were available ad libitum.

The animals were randomly divided into an experimental (n=10) and control (n=5) groups. Control group rats were untreated. Animals in the experimental group were anesthetized with pentobarbital sodium (40 mg/kg), and their bilateral ankle joints were fixed in full plantar flexion with plaster casts with the gastrocnemius muscles immobilized in a shortened position. Each plaster cast was positioned from above the knee joint to the distal foot was changed every 3 days to take account of loosening due to muscle atrophy. The rats in the experimental group were randomly partitioned into an ultrasound (US, n=5) and sham (n=5) treatment groups. For ultrasound or sham treatment, the bilateral ankle cast of each rat in the US and sham groups were removed under pentobarbital sodium anesthesia (40 mg/kg) 5 days per week during the 2-week immobilization period. Each treatment was performed for 15 min per day, 5 days per week over the 2-week immobilization period. Following completion of the daily treatment, bilateral ankle joints were re-immobilized via the aforementioned method.

In our previous study[2], continuous mode ultrasound irradiation (frequency, 1 MHz; intensity, 1.0 W/cm^2) applied to the triceps surae muscle of rats for 15 min increased the muscle temperature by approximately 6 °C. Moreover, approximately 7 min after the initiation of ultrasound, the muscle temperature rose to 40 °C. Subsequently, until the cessation of ultrasound treatment, the temperature of the triceps surae muscle of the rats scarcely changed for 8 min (at minimum). We consider the irradiation conditions of this ultrasonic wave are suitable for the induction of HSP 70 in skeletal muscles. Therefore, we used the same frequency, intensity and irradiation time as described in our previous report[2].

Therapeutic ultrasound was applied with an Ultrasound US-3 (Itoh Physio-therapy & Rehabilitation Ltd, Bunkyouku, Tokyo, Japan). The frequency was 1 MHz, beam non-uniformity ratio was 5.0, effective radiating area was 0.7 cm^2, area of applicator radiating surface was 0.785 cm^2 and the modulation frequency was 100 Hz ± 5%. Aqueous gel for ultrasound treatment served as the coupling medium. The triceps surae muscle in the US group was irradiated for 15 min with a continuous wave (intensity, 1.0 W/cm^2). In order to deliver ultrasonic irradiation as equally as possible to the entire muscle, the ultrasound transducer head was moved in a circular fashion over an area approximately twice the size of the effective radiating area. Ultrasonic energy was not delivered to the triceps surae muscle in the sham group; only the transducer head was moved. Ultrasound irradiation was applied 5 days per week over the 2-week immobilization period and the last irradiation was conducted two days before the end of the experimental period.

On the last day of the experimental period, all rats were anaesthetized with pentobarbital sodium (40 mg/kg), and the bilateral muscles of the gastrocnemius medialis were resected.

Right muscles were embedded in tragacanth gum, after which the samples were frozen in isopentane cooled by liquid nitrogen and stored in a deep freezer (−80 °C). Transverse serial sections (6 μm) cut from the belly of each muscle were mounted on glass slides. Some sections of muscle were stained with hematoxylin-eosin for histological observation. In order to classify muscle fiber types, myofibrillar adenosine triphosphatase (myosin ATPase) staining was performed. In the current study, the acid pre-incubation solution was adjusted to pH 4.5 to allow classification of the types of fibers of the gastrocnemius muscle as type I, IIa and IIb. The superficial gastrocnemius muscle is comprised almost entirely of type IIb fibers, whereas type I, IIa and IIb fibers are intermingled in the deep region of the gastrocnemius muscle[14]. The deep region of the gastrocnemius muscle was assessed in this investigation to examine the effects of continuous ultrasound irradiation on all fiber types. Light microscopy images of myosin ATPase staining were obtained with a digital camera (FUJIFILM HC-300Z, FUJIFILM, Minatoku, Tokyo, Japan) at 200× magnification. Next, muscle fiber diameters of each fiber type in the gastrocnemius muscles were measured employing the Scion image program for Windows (Scion Image Beta 4.0.3, Scion cooperation, Frederick, MD, USA) to analyze more than 100 fibers. The measurements were performed by two person blinded to the experimental groupings.

To determine HSP70 levels in muscles, polyacrylamide

Table 1. Mean muscle fiber diameters in gastrocnemius of each group

	control	US	sham
mean diameter of type I	31.9 ± 5.3	32.4 ± 5.5†	28.5 ± 5.4*
mean diameter of type IIa	35.8 ± 6.2	32.9 ± 4.8*†	30.8 ± 4.6*
mean diameter of type IIb	41.7 ± 7.5	36.4 ± 6.6*†	31.9 ± 5.6*

Data are means ± SD; * significant difference ($p<0.05$) compared with the control group. †significant difference ($p<0.05$) compared with the sham group.

Table 2. Contents of HSP70, bFGF, IGF-I of each group

	control	US	sham
HSP70 (% of control)	100.0 ± 43.1	160.8 ± 57.3†	81.2 ± 34.4
bFGF (pg/mg)	53.5 ± 17.3	156.4 ± 32.4*†	90.4 ± 34.2
IGF-I (pg/mg)	848.3 ± 138.6	802.2 ± 255.1	581.1 ± 173.3

Data are means ± SD; * significant difference ($p<0.05$) compared with the control group. †significant difference ($p<0.05$) compared with the sham group.

gel electrophoresis and western blotting were performed. Portions of the deep regions of the left muscles were homogenized in ice-cold homogenization buffer (10 mM Tris, 10 mM NaCl, 0.1 mM EDTA, pH 7.6). Homogenates were centrifuged at 4 °C at 12,000 revolutions/sec for 15 min,and the supernatant solutions were stored in a deep freezer (−80 °C). Following determination of total protein concentrations of the supernatants with a BCA Protein Assay Kit (PIERCE, Rockford, IL, USA), concentrations were adjusted to 1 mg/ml. SDS sample buffer (Bio-Rad Laboratories, Inc, Hercules, CA, USA) and 2-mercaptoethanol were added to the supernatants, which were then subjected to HSP70 analysis.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in order to separate the proteins by molecular weight. Protein from each muscle sample and pre-stained SDS-PAGE Standards (Bio-Rad Laboratories, Inc, Hercules, CA, USA) were loaded on the 12.5% polyacrylamide gel. Following electrophoretic separation, proteins were transferred to PVDF membranes. Subsequently, membranes were blocked for 1 h with TBS/Casein Blocker (Bio-Rad Laboratories, Inc, Hercules, CA, USA) with 0.05% Tween 20. The membranes were incubated for 2 h with a monoclonal antibody specific to HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 in TBS/Casein Blocker with 0.05% Tween 20. After washing three times (5 min each) in Tris-buffered saline with Tween 20, the blots were incubated with a secondary antibody (goat anti-mouse IgG-conjugated horseradish peroxidase, Vector Laboratories, Burlingame, CA, USA) for 2 h. After completion of several washings, the HSP70 bands were detected with a Metal Enhanced DAB Substrate Kit (PIERCE, Rockford, IL, USA). Immunoblots were captured with an image scanner (EPSON GT-X970, EPSON, Suwa, Nagano, Japan) and, quantification of the HSP70 bands was performed with the Scion image program for Windows.

Portions of the deep regions of the left muscles were homogenized in 0.01 M phosphate-buffered saline (PBS, pH 7.4). Homogenates were centrifuged at 4 °C at 10,000 revolutions/sec for 10 minand, the supernatant solutions were stored in a deep freezer (−80 °C). The supernatant solutions were used in enzyme-linked immunosorbent assays (ELISA) and, the amount of total protein in each supernatant of muscle was determined with a BCA Protein Assay Kit. bFGF and IGF-I levels in muscles were assayed with ELISA kits (Quantikine®, R&D System, Minneapolis, MN, USA) according to the instructions of the manufacturer. Briefly, the supernatants of the muscle sample were incubated on pre-coated microplates with bFGF or IGF-I for 2 h at room temperature (RT). After incubation, the microplates were washed and incubated with bFGF- or IGF-I-conjugated HRP for 2 h at RT. Subsequently, the microplates were washed and incubated with substrate solution for 30 min at RT in the dark. The reaction was terminated by adding sulfuric acid. Color development was monitored at 450 nm employing a microplate reader (Biotec, Bunkyouku, Tokyo, Japan) and the concentrations were calculated using the standard curves, as per protein (pg/mg).

All data are presented as mean ± S.D and were analyzed using one-way analysis of variance (ANOVA). When appropriate, Scheffe's test was performed for post hoc comparisons. Statistical significance was accepted at $p<0.05$.

RESULTS

Little myofiber necrosis was evident and tissue edema was not detected in muscle of any groups. The sham group displayed atrophic changes, including decreased fiber diameter, relative to the control group. Muscle fiber atrophy was obvious in the US group; however, muscle fiber atrophy of the US group was less than that of the sham group.

Mean muscle fiber diameters of all fiber types decreased significantly in the sham group in comparison with the control group. Similarly, mean muscle fiber diameters of type IIa and IIb in the US group decreased markedly relative to those of the control group. However, mean type I muscle fiber diameter of the US group did not differ from that of the

control group. The reductions in mean muscle fiber diameters of all types were significantly attenuated in the US group compared with the sham group (Table 1).The HSP70 content of the US group increased significantly in comparison with the sham group. bFGF levels of the US group were markedly higher than those of the control and sham groups. However, meaningful differences in IGF-I levels were not observed among the groups (Table 2).

DISCUSSION

Few studies documenting the effect of therapeutic ultrasound irradiation on atrophied skeletal muscle appear in the literature. Our present investigation assessed whether continuous therapeutic ultrasound inhibits the progression of disuse muscle atrophy in the rat gastrocnemius muscle. The present findings reveal that the mean diameters of all fiber types in the sham group decreased in comparison to the control group, and progression of muscle fiber atrophy induced by joint immobilization was clear. The mean diameters of type IIa and type IIb myofibers in the US group decreased relative to those of the control group; however, no meaningful difference was evident between the control and US groups in terms of the mean diameter of type I myofibers. In addition, mean diameters of all fiber types in the US group were markedly larger than those of the sham group. These results indicate that continuous mode therapeutic ultrasound irradiation can inhibit the progression of disuse muscle fiber atrophy induced by joint immobilization.

In general, therapeutic applications can be classified into two types: thermal and non-thermal ultrasound. Thermal ultrasound has several therapeutic effects, including increased blood flow velocity[15] and prevention of decrease in collagen fibril movement in the endomysium[2]. The mechanisms governing these beneficial effects remain the topic of debate. Elevated muscle temperature due to therapeutic ultrasound irradiation has been described in some previous reports. Draper et al.[16] noted that the triceps surae muscle temperature in humans increases by approximately 3.5 °C after 10 min exposure to ultrasound irradiation (frequency, 1 MHz; intensity, 1.0 W/cm^2, at 2.5 cm depth). Locke and Nussbaum[1] demonstrated that 15 min application of continuous mode ultrasound irradiation (frequency, 1 MHz; intensity, 1.0 W/cm^2) increased the temperature of rat triceps surae muscle by 6 °C. Additionally, the findings of our pilot study revealed that core temperature did not change; rather, peak muscle temperature increased by approximately 6 °C during ultrasound irradiation[2]. In the present investigation, the conditions of ultrasound irradiation were identical to those used in our previous study. Consequently, we consider the triceps surae muscles of the rats received heating sufficient to induce upregulation of HSP70 expression

HSP70 may play protective roles as chaperones in cellular stress[17]. These roles would include protein formation, maturation, and degradation, and the transport and assembly of newly formed proteins. Induction of HSP70 in skeletal muscle may result in the acceleration of protein synthesis and the inhibition of proteolysis, which may lead to inhibition of disuse muscle atrophy. Naito et al.[18] demonstrated that pretreatment of whole body with heat stress upregulated HSP70 8 days later in the rat soleus muscle and disuse muscle atrophy was attenuated in the heated-hindlimb suspension group. Furthermore, heat stress on alternate days during 8 days of hindlimb immobilization caused the elevation of HSP70 and the inhibition of disuse muscle atrophy in the rat soleus muscle[19]. Heat stress is the primary inducer of HSP70. Locke and Nussbaum[1, 8] reported that single-dose pulse and continuous ultrasound irradiation failed to induce HSP70 in rat gastrocnemius muscle. In contrast, continuous ultrasound irradiation for four consecutive days induced HSP70. Therefore, repeated continuous ultrasound irradiation may possibly inhibit the progression of disuse muscle atrophy. In the present study, HSP70 content in the gastrocnemius of the sham group was significantly lower than that of the control group. The HSP70 levels in the US group were significantly higher than those in the sham group. Moreover, these levels tended to be higher than those in the control group. However, no meaningful increase in HSP70 was detected between the two groups. These findings are consistent with the results of mean muscle fiber diameter. These data suggest that thermal ultrasound irradiation induces elevation of HSP70, leading to the inhibition of the progression of disuse muscle atrophy.

Therapeutic ultrasound exerts non-thermal effects, including the promotion of soft tissue repair[20]. Reher et al.[9] reported that non-thermal ultrasound irradiation of cultured osteoblasts increased production of bFGF and that this mechanism may be involved in the promotion of soft tissue wound repair. bFGF, which is the principal angiogenic factor, is thought to be a powerful regulator of skeletal muscle in vitro. Furthermore, bFGF may be associated with muscle hypertrophy and atrophy in vivo. Mitchell et al.[11] noted that stretch-induced hypertrophy led to an increase in bFGF mRNA levels in avian muscles and that bFGF localized the perimysium and endomysium of the myofiber periphery. Yamaguchi et al.[21] reported that the expression of bFGF mRNAs increased shortly after the initiation of compensatory overload and decreased consequent to denervation. Kästner et al.[22] described the enhancement of satellite cell proliferation upon the introduction of bFGF to cultured rat myofibers. In the present study, bFGF levels in the gastrocnemius decreased significantly in the sham group in comparison to the control group. On the other hand, bFGF level in the US group increased markedly by a factor of three relative to the control group. Clearly, ultrasound irradiation in thermal mode elevated bFGF levels in the rat gastrocnemius muscle. The difference in bFGF contents between the control and US groups was significant; however, the results of the enzyme-linked immunosorbent assays for bFGF were not consistent with the findings of the mean diameter of the myofibers. Tanaka et al.[23] stated that bFGF expression was not involved in muscle regeneration and hypertrophy given that expression patterns of bFGF mRNA were inconsistent with those of the myogenic or cell cycle markers, e.g., myoblast determination (MyoD) protein and proliferating cell nuclear antigen (PCNA), during hypertrophy induced by synergistic ablation. With respect to the results of this and earlier studies, bFGF expression induced

by ultrasound irradiation may exert a lesser effect than that of HSP expression in terms of inhibition of the progression of muscle atrophy due to joint immobilization.

In contrast to HSP70, IGF-I levels demonstrated no meaningful differences between the groups. Presumably, IGF-I is neither induced in skeletal muscle by continuous mode ultrasound irradiation nor is it involved in the inhibition of the progression of disuse muscle atrophy following ultrasound irradiation. IGF-I, which is structurally similar to proinsulin, stimulates cell regeneration and regulates metabolism. Additionally, IGF-I is the main growth factor involved in the stimulation of protein synthesis and promotes satellite cell activation in muscle tissue. Increases in muscle IGF-I mRNA have been observed during work-induced compensatory hypertrophy[24] and passive stretch-induced muscle growth in chicken[25] as well as in rabbit muscles[26]. In particular, IGF-I expressed in skeletal muscle following mechanical stress, e.g., resistance exercise or stretching, has been termed mechano growth factor (MGF). Mckoy et al.[27] reported that electrical stimulation with stretching could induce MGF in the extensor digitorum longus muscle of the rabbit, whereas electrical stimulation alone failed to induce MGF. As previously noted, IGF-I is upregulated by mechanical stress; therefore, we hypothesized that ultrasound irradiation would enhance IGF-I expression in the rat gastrocnemius. However, differences in IGF-I content were not evident between the groups. These findings are difficult to rationalize and their explanation will require further investigation.

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