平成 20 年度学位申請論文

Repetitive Stretching Prevents Muscle Atrophy in Denervated Soleus Muscle via Akt/mTOR/p70S6K Pathways

（周期的伸張刺激は Akt/mTOR/p70S6K 経路を介して除神経によるヒラメ筋萎縮を抑制する）

名古屋大学大学院医学系研究科

リハビリテーション療法学専攻

（指導：河上敬介　准教授）

縣　信秀

CONTENTS

Abstract

This study was conducted to examine whether stretch-related mechanical loading on skeletal muscle can suppress denervation-induced muscle atrophy, and if so, to depict the underlying molecular mechanism. Denervated rat soleus muscle was repetitively stretched (every 5 sec for 15 min/day) for 2 weeks. Histochemical analysis showed that the cross-sectional area of denervated soleus muscle fibers with repetitive stretching was significantly larger than that of control denervated muscle ($p<0.05$). We then examined the involvement of the Akt/mammalian target of the rapamycin (mTOR) cascade in the suppressive effects of repetitive stretching on muscle atrophy. Repetitive stretching significantly increased the Akt, p70S6K and 4E-BP1 phosphorylation in denervated soleus muscle compared to controls ($p<0.05$). Furthermore, repetitive stretching-induced suppression of muscle atrophy was fully inhibited by rapamycin, a potent inhibitor of mTOR. These results indicate that denervation-induced muscle atrophy is significantly suppressed by stretch-related mechanical loading of the muscle through upregulation of the Akt/mTOR signal pathway.

Key words: Akt, muscle atrophy, p70S6K, rapamycin, repetitive stretch

Introduction

Skeletal muscles change their size and mass in response to various environmental factors. Muscle fibers atrophy, and muscle strength decreases in hypodynamia, defined as reduced load-bearing and locomotor activity. Hypodynamia-induced atrophy has been shown to occur in a variety of situations, including denervation, prolonged bed rest, cast immobilization, and hindlimb suspension [9,14,20,21,27]. It has been reported that static stretching suppressed the reduction in the wet weight of the soleus muscle due to cast immobilization and hindlimb suspension [10,27,32]. Furthermore, muscle atrophy and weakness can be suppressed by exercising hypodynamic skeletal muscles [3,12]. We supposed that mechanical loading of muscle fibers generated by exercise may play an important role in suppressing muscle atrophy. Exercise generates dynamic mechanical loading arising from muscle contraction and relaxation. To our knowledge, however, there has been no report concerning the atrophy-suppressive effects of repetitive stretching, which mimics dynamic mechanical loading, on muscle atrophy.

In recent years, a number of studies have been conducted to assess the molecular mechanisms involved in muscle hypertrophy and suppression of muscle atrophy. The signaling cascade, Akt/mammalian target of the rapamycin (mTOR)/70-kDa ribosomal protein S6 kinase (p70S6K) and/or eukaryotic initiation factor 4E binding protein 1 (4E-BP1), has been reported to be involved in muscle hypertrophy stimulated by

insulin-like growth factor 1 (IGF-1) [23]. Furthermore, an elevation of Akt phosphorylation by exercise has been shown [24,25]. Exogenous expression of the constitutively active form of Akt prevented denervation-induced muscle atrophy [7]. Thus it is highly likely that Akt activation is involved in the exercise-related suppression of muscle atrophy. It is also known that stretching of skeletal muscles activates Akt *via* phosphatidylinositol 3-kinase (PI3K) *ex vivo* [19,25], and that stretching and exercise activate p70S6K and 4E-BP1, the target protein of mTOR *in vivo* and *ex vivo* [8,19]. These findings suggest that mechanical loading applied to skeletal muscles activates the Akt/mTOR/p70S6K and/or 4E-BP1 signal cascade, and that Akt activation is sufficient to suppress muscle atrophy. However, it is not clear whether activation of the Akt/mTOR/p70S6K and/or 4E-BP1 signal cascade in response to stretch-related mechanical loading is indispensable in suppressing hypodynamia-induced muscle atrophy. To address this question, we examined whether repetitive stretching mimicking exercise-related tension bearing in the muscle would suppress atrophy in denervated muscles. In addition we assessed the involvement of the Akt/mTOR/p70S6K and/or 4E-BP1 signal cascade in the suppression of atrophy by repetitive stretching.

Materials and Methods

Animals and experimental design

All experiments were approved by the Animal Care Committee of the Nagoya University Graduate School of Medicine and followed the guiding principles for care and use of animals set by the Physiological Society of Japan.

Experiment 1**:** To investigate whether repetitive stretching suppresses denervation-induced muscle atrophy, 24 male Wistar rats (weight, 251 ± 12 g) were used. Animals were provided food and water *ad libitum* and were subjected to a 12-hour light-dark cycle. The animals were randomly divided into three groups of 8 animals each. In the control group (Con), the soleus muscle was subjected to sham operation with no sciatic nerve removed. In the denervated muscle group (Den), the soleus muscle was subjected to the denervation procedure with left sciatic nerve removal. In the stretching group (Str), the soleus muscle was subjected to the denervation procedure with left sciatic nerve removal, and the denervated soleus muscle underwent repetitive stretching for 15 min/day for 2 weeks, beginning 24 h after denervation.

Experiment 2**:** To demonstrate whether repetitive stretching increases Akt, p70S6K and 4E-BP1 phosphorylation in denervated muscle, 48 male rats (weight, 255 ± 8 g) were used. All rats were subjected to the denervation procedure with left sciatic

nerve removal. The animals were randomly divided into six groups of 8 animals each. In the sedentary group (Sed), the denervated soleus muscle was sedentary. In the stretching groups (Str 0, 5, 15, 30, 60), the denervated soleus muscles were subjected to repetitive stretching for 15 min at 7 days after denervation, and they were evaluated at time intervals of 0, 5, 15, 30, and 60 min after stretching.

Experiment 3: First, to examine whether rapamycin suppresses the stretch induced p70S6K phosphorylation through an inhibiton of mTOR without affecting Akt phosphorylation, 12 male rats (250 ± 10 g) were used. All rats were subjected to the denervation procedure with bilateral sciatic nerve removal. The animals were randomly divided into a rapamycin treatment group and an excipient group of 6 animals each. At 7 days after denervation, rats in the rapamycin treatment group were administered 0.75 mg rapamycin /kg body weight (Calbiochem Novabiochem, La Jolla, CA, USA) via tail vein as previously described [4]. The rats of the excipient group were administered an equal volume of excipient (0.155 mol/L NaCl, 2% v/v ethanol). Two hours after rapamycin or excipient administration, the left soleus muscles in both groups of rats were subjected to repetitive stretching for 15 min. The right soleus muscles were sedentary. All muscles were examined immediately after the end of the repetitive stretching period. Next, to demonstrate whether activation of the mTOR pathway is indispensable in the suppression of muscle atrophy by repetitive stretching, 16 male

rats (weight, 254 ± 12 g) were used. All rats were subjected to the denervation procedure with bilateral sciatic nerve removal. The animals were randomly divided into two groups of 8 animals each. The rats of the denervated group receiving rapamycin (Den+Rap) were administered 0.75 mg rapamycin /kg body weight *via* the tail vein. In the denervated group (Den), the rats were administered an equal volume of excipient (0.155 mol/L NaCl, 2% v/v ethanol). Two hours later, the left soleus muscles in the Den+Rap and Den groups were subjected to repetitive stretching for 15 min/day for 2 weeks, which began 24 h after denervation (Den+Rap+Str, Den+Str). The right soleus muscles were sedentary.

Denervation Procedure

The rats were anesthetized with an intraperitoneal injection of sodium pentobarbitone (40 mg/kg) for the denervation processes. A small incision was made through the skin and fascia near the trochanter between the gluteus maximus and biceps femoris muscles. The muscles were separated to isolate the sciatic nerve, and about 2 cm of the nerve was cut and removed. The fascia and skin were then sutured with silk thread.

Stretching technique

Repetitive stretching of the soleus muscle was performed under anesthesia. The rats were placed in the lateral recumbent position and subjected to manual passive

manipulation of the ankle joint between neutral and maximal dorsiflexion while the knee was kept in extension every 5 sec for 15 min. We recorded the angle between a line from the caput fibulae to the lateral malleolus and a line from basis ossis metatarsalis to caput metatarsalis in the stretching period. The angle was approximately 70 degrees in the neutral position, and approximately 0 degrees in the maximal dorsiflexion position. The soleus muscle was chosen for this study because it crosses only the ankle joint, and histological cross-sections taken from the middle belly contain all muscle fibers, avoiding sampling problems. Additionally, this muscle has been widely used in previous studies of stretch on skeletal muscle [10,29,32].

Muscle preparation

At the end of experimental period, all rats were killed by cervical dislocation. The soleus muscles for histological analysis were quickly dissected and immediately frozen in isopentane, pre-cooled in liquid nitrogen, and stored in a freezer at -80°C. The soleus muscles for biochemical analyses were also quickly dissected, immediately frozen in liquid nitrogen, and stored at -80°C.

Measurement of fiber cross-sectional area

Cross-sections (8 μm) were obtained from the middle belly of the frozen muscles using a cryostat microtome and stained with hematoxylin and eosin. The cross-sectional area of 100 muscle fibers chosen randomally in the central region of

one cross-section of each soleus muscle was measured using a light microscope and Scion Image software (Scion Corp., Frederick, MD, USA) for morphology.

Western blot analysis

Samples were minced and homogenized in ice-cold homogenization buffer [20 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetate, 1% sodium dodecylsulfate (SDS), 25 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μM leupeptin, 5 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride]. Homogenates were centrifuged at 12,000 g for 15 min at 4°C, and the protein concentration of the supernatants was determined by using the micro BCA^{TM} protein assay (Pierce, Rockford, IL, USA). Samples were solubilized in a sample loading buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue] at 5 mg/ml and incubated at 60°C for 10 min. Proteins were then separated by 8% or 10% SDS-PAGE and subjected to Western blotting for 60 min onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After protein transfer, the membranes were blocked for 1 h at room temperature in blocking buffer [5% skim milk in phosphate-buffered saline (PBS)]. After serial washes with PBS, the membranes were incubated with primary antibodies to phosphorylated Ser473-Akt (diluted 1:1000 in 1% BSA in PBS; Cell Signaling, Beverly, MA, USA), Akt (1:1000; Cell Signaling), phosphorylated Thr389-p70 S6 kinase (p70S6K, 1:1000; Cell

Signaling), S6K1 (1:1000; Chemicon International, Temecula, CA, USA), phosphorylated Thr37/46-4E-BP1 (1:1000; Cell Signaling), or 4E-BP1 (1:1000; Cell Signaling) overnight at 4°C. After several washes with PBS, membranes were incubated with goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibodies (1:3000; Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at room temperature. An AP-substrate kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect protein signals, and band intensities were quantified by densitometry.

Statistical analysis

All data were reported as mean ± standard deviation. In Experiments 1 and 2, multigroup comparisons were performed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. In Experiment 3, data were compared by one-way ANOVA for repeated measures followed by the Bonferroni post hoc test. For all comparisons, the level of statistical significance was set at 5% ($P<0.05$).

Results

Repetitive stretching suppresses denervation-induced muscle atrophy

Denervated soleus muscle was repetitively stretched 15 min/day for 2 weeks. Immediately after the end of the experiment, the soleus muscle was removed, and the cross-sectional area of individual muscle fibers was measured. The average muscle

fiber area for the group without denervation (Con group) was 2446 ± 252 μm^2, while that for the group 2 weeks after denervation (Den group) was 790 ± 147 μm^2 (Fig. 1). The average muscle fiber area for the group with denervation and repetitive stretching (Str group) was 1127 ± 128 μm^2 (Fig. 1), which was significantly greater than that of the Den group ($p<0.05$). These results clearly demonstrate that repetitive stretching suppressed significantly denervation-induced muscle atrophy.

Repetitive stretching increases Akt, p70S6K and 4E-BP1 phosphorylation in denervated muscle

First, we compared the phosphorylation levels of Akt, p70S6K, 4E-BP1 in innervated soleus muscle with those of denervated soleus muscle in the sedentary condition. The phosphorylation levels of Akt, p70S6K, and 4E-BP1 in innevated soleus muecle increased by 2.0-fold, 2.8-fold, and 1.5-fold, respectively, with respect to those in denervated soleus muscle ($p<0.05$, data not shown). Next, the effects of repetitive stretching on Akt, p70S6K and 4E-BP1 phosphorylation levels in denervated soleus muscle were investigated. At 7 days after denervation, the muscle was subjected to repetitive stretching for 15 minutes and then was dissected 0, 5, 15, 30, or 60 minutes after the stretching period. Western blotting was performed to assess the phosphorylation levels of Akt, p70S6K and 4E-BP1. The phosphorylation levels of Akt

and p70S6K at 15 and 5 minutes after stretching increased by approximately 3.0-fold ($p<0.05$; Fig. 2A) and 2.3-fold ($p<0.05$; Fig. 2B), respectively. The level of 4E-BP1 phosphorylation at 15 and 60 minutes after repetitive stretching increased by 2.5-fold and 2.9-fold, respectively ($p<0.05$; Fig. 2C). These results indicate that repetitive stretching increased the level of Akt, p70S6K and 4E-BP1 phosphorylation in denervated muscle.

Rapamycin hinders the suppressive effects of repetitive stretching on muscle atrophy

We examined whether rapamycin suppresses stretch-induced p70S6K phosphorylation through an inhibition of mTOR without affecting Akt phosphorylation. At 7 days after denervation, rapamycin was administred, and the soleus muscles were subjected to repetitive stretching for 15 minutes, followed by immediate dissection. Then western blotting was performed to assess the phosphorylation levels of Akt and p70S6K. The levels of Akt phosphorylation with repetitive stretching and excipient, and rapamycin administration increased 1.9-fold and 2.0-fold, respectively ($p<0.05$; Fig. 3Aa). Meanwhile, the level of p70S6K phosphorylation in soleus muscle with repetitive stretching and excipient administration increased 2.8-fold ($p<0.05$; Fig. 3Ab), whereas that of soleus muscle with repetitive stretching and rapamycin remained unchanged (1.0-fold). Rapamycin suppressed the increase in the phosphorylation of

p70S6K by repetitive stretching, but it did not inhibit the stretch-induced increase in Akt phosphorylation. Next, to investigate whether the activation of mTOR pathway by repetitive stretching is indispensable in suppressing muscle atrophy, an experiment was conducted using the mTOR inhibitor rapamycin. Denervated soleus muscle was repetitively stretched for 15 min/day for 2 weeks. Rapamycin (0.75 mg/kg) was administered at 2 h before stretching. After the end of the experiment, the soleus muscle was dissected to measure the area of its muscle fibers (Fig. 3C). The average muscle fiber area for the group with denervation, rapamycin administration, and repetitive stretching (Den+Rap+Str group) was 1147 ± 177 μm^2, which was significantly smaller than that for the vehicle group (Den+Str group: 1566 ± 280 μm^2, $p<0.05$). No significant difference was found between the denervated groups with and without rapamycin administration (Den group: 1154 ± 36 μm^2; Den+Rap group: 1109 ± 177 μm^2), whereas for the denervated groups with repetitive stretching, there was a significant difference between the group with and without rapamycin (Den+Str group: 1566 ± 280 μm^2; Den+Str+Rap group: 1147 ± 177 μm^2). These results strongly suggest that activation of the mTOR cascade is indispensable for the suppressive effects of repetitive stretching on denervation-induced muscle atrophy.

Discussion

Hypodynamia-induced atrophy is suppressed by exercise [3,12]. This suppression is believed to involve many complicated factors, such as neural factors, hormones, and mechanical loads. Here, we have examined the effect of passive exercise on hypodynamic muscle by focusing on mechanical loads. It has been reported that hypodynamia-induced atrophy could be suppressed by static stretching *in vivo* [10,27,32]. However, whether repetitive stretching can suppress muscle atrophy is not known. Repetitive stretching applied to cultured skeletal muscle cells caused hypertrophy *in vitro* [1,31]. Muscle hypertrophy is thought to be caused by increased protein synthesis and/or suppressed protein degradation in myocytes. As in the case of muscle hypertrophy, suppression of muscle atrophy is thought to be caused by increased protein synthesis and/or suppressed protein degradation in myocytes.

The Akt/mTOR/p70S6K or 4E-BP1 cascade is one of the signaling mechanisms involved in increased protein synthesis [13]. *In vitro* and *ex vivo* studies have revealed that skeletal muscle stretching activates these signal molecules (Akt, mTOR, p70S6K, 4E-BP1) [5,19,25]. The present study also provides evidence that *in vivo* repetitive stretching of the denervated soleus muscle increases phosphorylation levels of Akt, p70S6K, and 4E-BP1. Furthermore, administration of rapamycin completely negated the suppressive effects of repetitive stretching on muscle atrophy (Fig. 3). The present

study is the first to demonstrate that the Akt/mTOR pathway is indispensable in the suppressive effects of repetitive stretching on muscle atrophy *in vivo*.

The exogenous expression of the constitutively active form of Akt alone prevents denervation-induced atrophy, suggesting that Akt activation is sufficient to prevent muscle atrophy [7]. Akt activation is known not only to facilitate muscle protein synthesis *via* mTOR, but also to suppress protein degradation by controlling ubiquitin ligase activity *via* the phosphorylation of the forkhead-related transcription factor FOXO [6,26,30]. Therefore, suppression of muscle atrophy by mechanical loading has been suggested to involve the suppression of muscle protein degradation *via* the modification of ubiquitin ligase activity. Recently, it has been reported that the kinase domain of titin (connectin), a giant elastic protein which spans from the Z line to a thick filament, strongly binds with the muscle specific RING finger protein MuRF that controls ubiquitin ligase activity [16]. Furthermore, the kinase domain of titin has been suggested to be involved in the reception of mechanical stimulation [15]. These findings suggest that suppression of muscle protein degradation through ubiquitin ligase activity would play a crucial role in the suppressive effects of mechanical loading on muscle atrophy. However, the present study revealed that the suppressive effects of repetitive stretching were completely negated by rapamycin, an inhibitor of mTOR that is downstream of Akt. Thus, the suppressive effects of repetitive stretching on muscle

atrophy are mostly dependent on increased protein synthesis rather than decreased protein degradation in our model.

The calcineurin/nuclear factor of activated T cell (NFAT) is another possible signal cascade involved in the suppression of muscle atrophy by stretching. It was reported that the calcineurin/NFAT cascade, which is activated by an increase in the intracellular calcium ion concentration, was involved in myocardial hypertrophy [18]. However, some studies reported that the calcineurin/NFAT cascade was not involved in skeletal muscle hypertrophy [11,22,23], thus the issue remains controversial. In the present study, rapamycin almost completely negated the suppressive effects of repetitive stretching on muscle atrophy, suggesting that NFAT activation may not contribute to the suppression of muscle atrophy in our experimental system.

The molecular mechanisms of Akt activation by skeletal muscle stretching remain mostly unknown. Although stretching or contracting skeletal muscles increases the expression of IGF-1 mRNA in myocytes to elevate the concentration of IGF-1 in myocytes [2,33], it is unclear whether the stretching-induced IGF-1 increase in myocytes activates Akt *via* autocrine/paracrine mechanism. Mechanical loading to vascular smooth muscle cells activates Akt *via* an integrin-dependent pathway [28]. Integrins accumulate in costameres, an anchoring apparatus that transmits tension generated by the actin-myosin interaction to the basal membrane and focal adhesion in striated

muscle [17]. Therefore, it is possible that Akt is directly activated *via* integrin-dependent intracellular signaling pathways in skeletal muscle. However, further investigations are needed to elucidate the mechanisms underlying the stretch-dependent Akt activation in skeletal muscle.

Reference List

1. Adachi R, Yabusaki K, Obinata T. Uptake of albumin is coupled with stretch-induced hypertrophy of skeletal muscle cells in culture. Zoolog Sci 2003; 20: 557-565.

2. Adams GR. Invited Review: Autocrine/paracrine IGF-I and skeletal muscle adaptation. J Appl Physiol 2002; 93: 1159-1167.

3. Alkner BA, Tesch PA. Knee extensor and plantar flexor muscle size and function following 90 days of bed rest with or without resistance exercise. Eur J Appl Physiol 2004; 93: 294-305.

4. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr 2000; 130: 2413-2419.

5. Baar K, Torgan CE, Kraus WE, Esser K. Autocrine phosphorylation of p70(S6k) in response to acute stretch in myotubes. Mol Cell Biol Res Commun 2000; 4: 76-80.

6. Bodine SC. mTOR signaling and the molecular adaptation to resistance exercise. Med Sci Sports Exerc 2006; 38: 1950-1957.

7. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. Akt/mTOR pathway is a crucial regulator of

skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 2001; 3: 1014-1019.

8. Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR, Jefferson LS. Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle. J Physiol 2003; 553: 213-220.

9. Boonyarom O, Inui K. Atrophy and hypertrophy of skeletal muscles: structural and functional aspects. Acta Physiol (Oxf) 2006; 188: 77-89.

10. Coutinho EL, Gomes AR, Franca CN, Oishi J, Salvini TF. Effect of passive stretching on the immobilized soleus muscle fiber morphology. Braz J Med Biol Res 2004; 37: 1853-1861.

11. Dupont Salter AC, Richmond FJ, Loeb GE. Prevention of muscle disuse atrophy by low-frequency electrical stimulation in rats. IEEE Trans Neural Syst Rehabil Eng 2003; 11: 218-226.

12. Fluckey JD, Dupont-Versteegden EE, Montague DC, Knox M, Tesch P, Peterson CA, Gaddy-Kurten D. A rat resistance exercise regimen attenuates losses of musculoskeletal mass during hindlimb suspension. Acta Physiol Scand 2002; 176: 293-300.

13. Glass DJ. Molecular mechanisms modulating muscle mass. Trends Mol Med 2003; 9: 344-350.

14. Goldspink DF. The effects of denervation on protein turnover of rat skeletal muscle. Biochem J 1976; 156: 71-80.

15. Grater F, Shen J, Jiang H, Gautel M, Grubmuller H. Mechanically induced titin kinase activation studied by force-probe molecular dynamics simulations. Biophys J 2005; 88: 790-804.

16. Gregorio CC, Perry CN, McElhinny AS. Functional properties of the titin/connectin-associated proteins, the muscle-specific RING finger proteins (MURFs), in striated muscle. J Muscle Res Cell Motil 2005; 26: 389-400.

17. Grounds MD, Sorokin L, White J. Strength at the extracellular matrix-muscle interface. Scand J Med Sci Sports 2005; 15: 381-391.

18. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 2006; 7: 589-600.

19. Hornberger TA, Stuppard R, Conley KE, Fedele MJ, Fiorotto ML, Chin ER, Esser KA. Mechanical stimuli regulate rapamycin-sensitive signalling by a phosphoinositide 3-kinase-, protein kinase B- and growth factor-independent mechanism. Biochem J 2004; 380: 795-804.

20. LeBlanc A, Gogia P, Schneider V, Krebs J, Schonfeld E, Evans H. Calf muscle area and strength changes after five weeks of horizontal bed rest. Am J Sports Med 1988; 16: 624-629.

21. Nicks DK, Beneke WM, Key RM, Timson BF. Muscle fibre size and number following immobilisation atrophy. J Anat 1989; 163: 1-5.

22. Parsons SA, Millay DP, Wilkins BJ, Bueno OF, Tsika GL, Neilson JR, Liberatore CM, Yutzey KE, Crabtree GR, Tsika RW, Molkentin JD. Genetic loss of calcineurin blocks mechanical overload-induced skeletal muscle fiber type switching but not hypertrophy. J Biol Chem 2004; 279: 26192-26200.

23. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 2001; 3: 1009-1013.

24. Sakamoto K, Arnolds DE, Ekberg I, Thorell A, Goodyear LJ. Exercise regulates Akt and glycogen synthase kinase-3 activities in human skeletal muscle. Biochem Biophys Res Commun 2004; 319: 419-425.

25. Sakamoto K, Aschenbach WG, Hirshman MF, Goodyear LJ. Akt signaling in skeletal muscle: regulation by exercise and passive stretch. Am J Physiol Endocrinol Metab 2003; 285: E1081-E1088.

26. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and

cause skeletal muscle atrophy. Cell 2004; 117: 399-412.

27. Sasa T, Sairyo K, Yoshida N, Fukunaga M, Koga K, Ishikawa M, Yasui N. Continuous muscle stretch prevents disuse muscle atrophy and deterioration of its oxidative capacity in rat tail-suspension models. Am J Phys Med Rehabil 2004; 83: 851-856.

28. Sedding DG, Hermsen J, Seay U, Eickelberg O, Kummer W, Schwencke C, Strasser RH, Tillmanns H, Braun-Dullaeus RC. Caveolin-1 facilitates mechanosensitive protein kinase B (Akt) signaling in vitro and in vivo. Circ Res 2005; 96: 635-642.

29. Stauber WT, Miller GR, Grimmett JG, Knack KK. Adaptation of rat soleus muscles to 4 wk of intermittent strain. J Appl Physiol 1994; 77: 58-62.

30. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 2004; 14: 395-403.

31. Vandenburgh H, Kaufman S. In vitro model for stretch-induced hypertrophy of skeletal muscle. Science 1979; 203: 265-268.

32. Williams PE. Use of intermittent stretch in the prevention of serial sarcomere loss in immobilised muscle. Ann Rheum Dis 1990; 49: 316-317.

33. Yang H, Alnaqeeb M, Simpson H, Goldspink G. Changes in muscle fibre type, muscle mass and IGF-I gene expression in rabbit skeletal muscle subjected to stretch. J Anat 1997; 190 (Pt 4): 613-622.

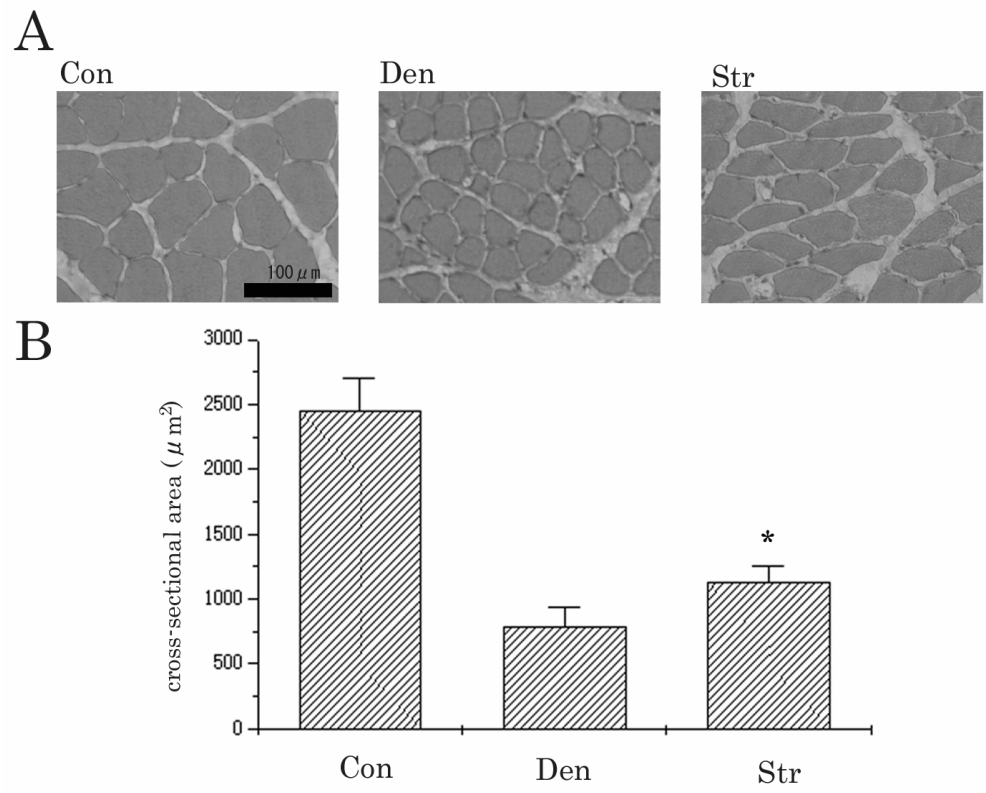
A
Con
Den
Str
100 μm
B
cross-sectional area (μm²)
3000
2500
2000
1500
1000
500
0
*
Con
Den
Str

Figure 1. **Effect of repetitive stretching on fiber area of denervated soleus muscle.**

A: Bright fields images of muscle fiber cross-sections of the soleus muscle stained with H-E. Con, the soleus muscle was subjected to sham operation with no sciatic nerve removed; Den, denervated soleus muscle; Str, denervated soleus muscle subjected to repetitive stretching. Bar, 100 μm. B: Cross-sectional area of soleus muscle fibers. The data are mean ± standard deviation. * $P<0.05$ when the Str group was compared to the Den group.

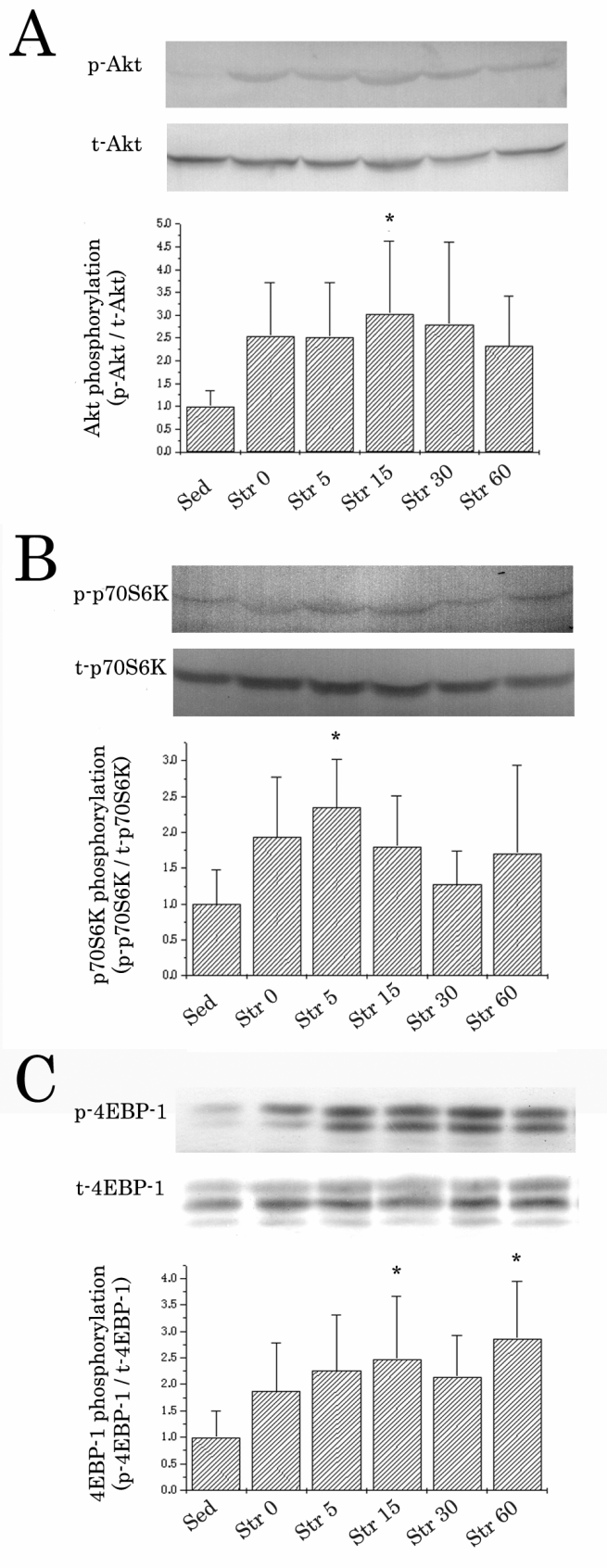
A
p-Akt
t-Akt
Akt phosphorylation
(p-Akt / t-Akt)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Sed
Str 0
Str 5
Str 15
Str 30
Str 60
B
p-p70S6K
t-p70S6K
p70S6K phosphorylation
(p-p70S6K / t-p70S6K)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Sed
Str 0
Str 5
Str 15
Str 30
Str 60
C
p-4EBP-1
t-4EBP-1
4EBP-1 phosphorylation
(p-4EBP-1 / t-4EBP-1)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Sed
Str 0
Str 5
Str 15
Str 30
Str 60

Figure 2. Effect of repetitive stretching on Akt, p70S6K and 4E-BP1 phosphorylation in denervated soleus muscle.

Representative Western blot used to determine phosphorylated Akt/Akt levels (A, *Top*), phosphorylated p70S6K/p70S6K levels (B, *Top*), and phosphorylated 4E-BP1/4E-BP1 levels (C, *Top*) in soleus muscles of all six experimental groups. Comparative levels of phosphorylaed Akt/Akt (A, *Bottom*), phosphorylated p70S6K/p70S6K (B, *Bottom*), and phosphorylated 4E-BP1/4E-BP1 levels (C, *Bottom*) across experimental groups, expressed as fold increase of sedentary soleus muscle (Sed) versus stretched soleus muscle (Str 0, 5, 15, 30, 60) submitted to repetitive stretching for 15 min and evaluated immediately afterwards (0) or 5, 15, 30, and 60 min later. *$P<0.05$ compared to the Sed group.

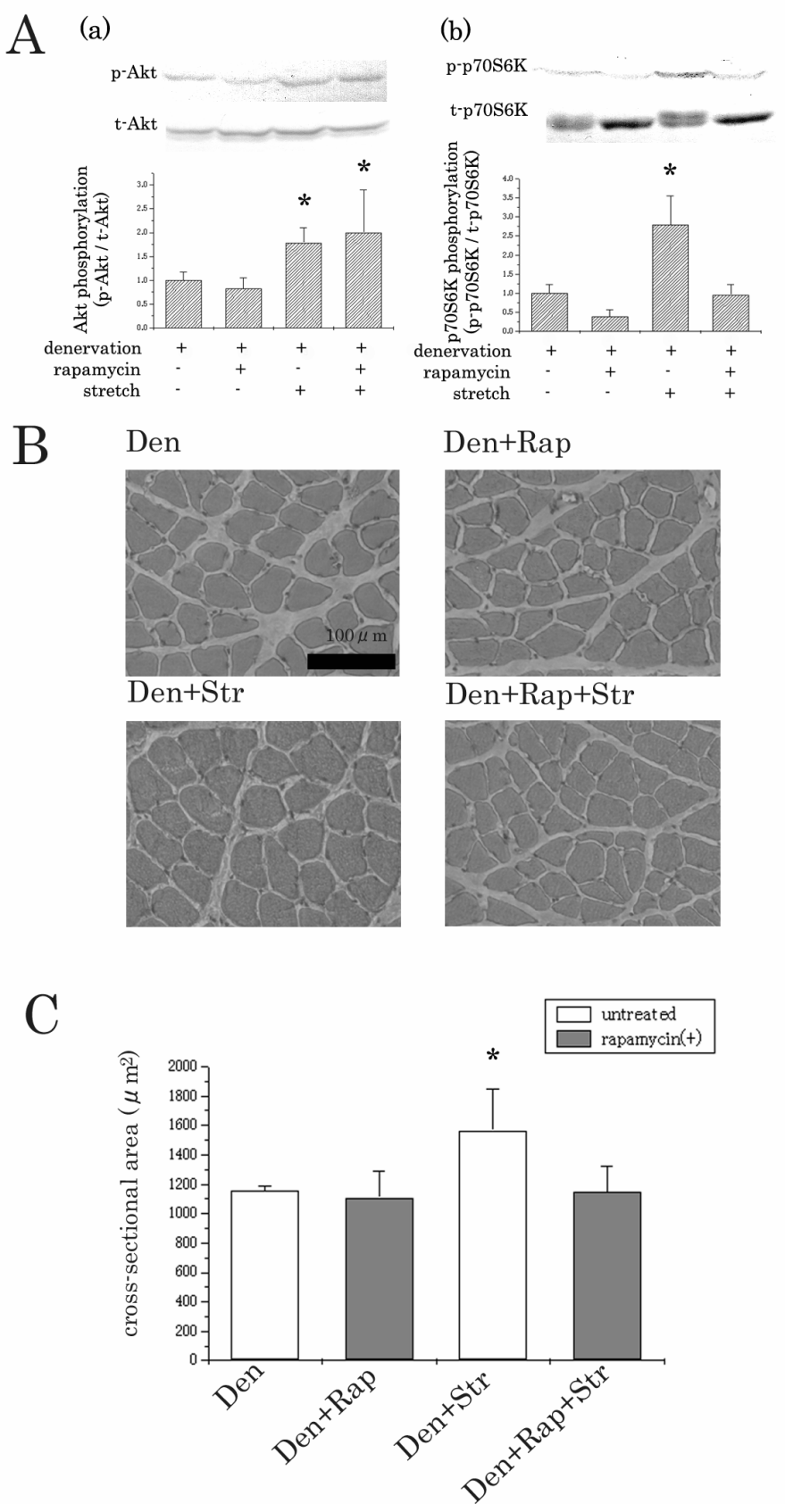
A
(a)
p-Akt
t-Akt
Akt phosphorylation (p-Akt / t-Akt)
denervation + + + +
rapamycin - + - +
stretch - - + +
(b)
p-p70S6K
t-p70S6K
p70S6K phosphorylation (p-p70S6K / t-p70S6K)
denervation + + + +
rapamycin - + - +
stretch - - + +
B
Den
Den+Rap
100 μm
Den+Str
Den+Rap+Str
C
untreated
rapamycin(+)
cross-sectional area (μm²)
Den
Den+Rap
Den+Str
Den+Rap+Str

Figure 3. Effect of rapamycin on fiber area of denervated soleus muscle.

Representative Western blot used to determine the phosphorylated Akt/Akt levels (Aa, *Top*) and phosphorylated p70S6K/p70S6K levels (Ab, *Top*) in soleus muscles of all four experimental groups. Comparative levels of phosphorylaed Akt/Akt (Aa, *Bottom*) and phosphorylated p70S6K/p70S6K (Ab, *Bottom*) across experimental groups, expressed as fold increase of denervated soleus muscle with not stretching and excipient administration versus any other group of soleus muscles. B: Muscle fiber cross-sections of the soleus muscle stained with H-E. Den, denervated soleus muscle in the rat administered excipient (0.155 mol/L NaCl, 2% v/v ethanol); Den+Rap, denervated soleus muscle in the rat administered 0.75 mg rapamycin /kg body weight; Den+Str, denervated soleus muscle subjected to repetitive stretching in the rat administered excipient; Den+Rap+Str, denervated soleus muscle subjected to repetitive stretching in the rat administered 0.75 mg rapamycin. Bar, 100 μm. C: Cross-sectional area of soleus muscle fibers. The data are mean ± standard deviation. * $P<0.05$ when the Den+Str group was compared to any other group.

和文抄録

Repetitive Stretching Prevents Muscle Atrophy in Denervated Soleus Muscle via Akt/mTOR/p70S6K Pathways

（周期的伸張刺激は Akt/mTOR/p70S6K 経路を介して除神経によるヒラメ筋萎縮を抑制する）

名古屋大学大学院医学系研究科
リハビリテーション療法学専攻
縣　信秀　(指導: 河上　敬介　准教授)

本研究の目的は、運動負荷時に骨格筋に加わる張力による、筋萎縮軽減のメカニズムを明らかにすることである。まず、ラット除神経ヒラメ筋に対して、除神経翌日から周期的伸張刺激を 1 日 15 分間、2 週間行い、筋萎縮軽減効果を調べた。その結果、1 日 15 分間の周期的伸張刺激を加えた除神経ヒラメ筋の筋線維断面積（1127±128 μm²）は、除神経後 2 週間のヒラメ筋の筋線維断面積（790±147 μm²）に比べ有意に大きかった（$p<0.05$）。このことから、周期的伸張刺激によって除神経による筋萎縮が軽減されることが明らかになった。これまでに、IGF-1 刺激による筋肥大に Akt/mTOR/p70S6K and/or 4E-BP1 経路 が関与していることや、*ex vivo* において、骨格筋に対する伸張刺激が Akt を活性化することが報告されている。そこで、周期的伸張刺激による筋萎縮軽減に、Akt/mTOR/p70S6K and/or 4E-BP1 経路が関与しているかどうかを調べた。その結果、除神経ヒラメ筋に対して、15 分間の周期的伸張刺激を加えると、Akt、p70S6K、4E-BP1 のリン酸化が有意に亢進することがわかった（$p<0.05$）。さらに、mTOR の阻害剤である rapamycin によって周期的伸張刺激による筋萎縮軽減効果は抑制された。以上のことから、本研究により運動負荷時に加わるような周期的な張力が骨格筋の萎縮を軽減すること、そのメカニズムに Akt/mTOR/p70S6K and/or 4E-BP1 経路が大きく関わることが明らかになった。