平成 21 年度学位申請論文

Involvement of PI3K/Akt/TOR pathway in stretch-induced hypertrophy of myotubes

（伸張刺激により引き起こされる筋管細胞肥大への PI3K/Akt/TOR 経路の関与）

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）

笹井　宣昌

CONTENTS

Abstract

Skeletal muscle cells are hypertrophied by mechanical stresses, but the underlying molecular mechanisms are not fully understood. Two signaling pathways, phosphatidylinositol 3-kinase (PI3K)/Akt to target of rapamycin (TOR) and extracellular signal-regulated kinase kinase (MEK) to extracellular signal-regulated kinase (ERK), have been proposed to be involved in muscle hypertrophy. In this study we examined the involvement of these pathways in primary cultures of chick skeletal myotubes subjected to passive cyclic stretching for 72 hours, a time that was sufficient to induce significant hypertrophy in our preparations. Hypertrophy was largely suppressed by wortmannin or rapamycin, inhibitors of PI3K or mTOR, respectively. Furthermore, phosphorylation of Akt was enhanced by stretching and suppressed by wortmannin. The MEK inhibitor, U0126, exerted a minimal influence on stretch-induced hypertrophy. We found that cyclic stretching of myotubes activates the PI3K/Akt/TOR pathway, resulting in muscle hypertrophy. The MEK/ERK pathway may contribute negatively to spontaneous hypertrophy.

Key words: Akt, cultured cells, cyclic stretching, ERK, muscle hypertrophy

Introduction

Hypertrophy of skeletal muscle is induced by mechanical loading such as high-intensity resistance training.[9] *In vitro* studies showed that passive stretching promotes hypertrophy of cultured muscle cells[1,33]; however, the molecular mechanisms underlying mechanically -induced hypertrophy are poorly understood. Clarification of the molecular mechanisms that regulate skeletal muscle hypertrophy is essential for providing strategies to optimize and maintain skeletal muscle mass.[7]

As a possible mechanism that regulates muscle mass, the phosphatidylinositol 3-kinase (PI3K)/Akt/target-of-rapamycin (TOR) pathway is well recognized.[10,11] Generally, this pathway is activated by insulin-like growth factor-1 (IGF-1) and plays a crucial role in cell growth and survival. Its down-stream effectors are thought to be involved in translational regulation of protein synthesis.[11,19,25,31] In muscle, the constitutive activation of Akt is sufficient to suppress atrophy.[6] Akt suppresses protein degradation caused by muscle-specific ring finger proteins[28,30], thus leading to the hypertrophy induced by IGF-1.[6,25] Involvement of PI3K/Akt/TOR signaling in mechanically induced muscle hypertrophy has not been clearly demonstrated except for the case of mechanically prevented muscle atrophy *in vivo*.[3] Besides the PI3K/Akt/TOR pathway, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated

kinase kinase (MEK) / extracellular signal-regulated kinase (ERK) pathway is involved in the regulation of muscle mass.[27] ERK is a member of the MAPK family and is activated directly by MEK in a variety of cellular functions, including proliferation, differentiation, and survival.[17] In skeletal muscle, these molecules work in β-agonist-induced hypertrophy[27] and are activated by mechanical stress.[21] Furthermore, in cardiac muscle, which is striated like skeletal muscle, activation of the MAPK pathway by mechanical stress regulates hypertrophy-related gene expression, thus leading to cardiac hypertrophy.[34,36] However, it is unclear whether the Akt and MAPK pathways are essential for mechanically induced skeletal muscle hypertrophy. To address this issue, we designed an *in vitro* experiment, in which primary cultured chick skeletal myotubes on an elastic silicone membrane were subjected to cyclic stretching to induce hypertrophy, and the involvement of those signaling pathways was examined by pharmacologic and biochemical techniques.

Materials and Methods

Experimental procedures 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.

Cell culture. Primary cultures of mononucleated myogenic cells were prepared from breast muscles of 13-day-old chicken embryos by means of mechanical dissociation.[16] Cells were sowed onto collagen-coated culture ware at a density of 8.4-11.1 × 10^4 cells/cm^2; the collagen coating was dispensed with 0.05% collagen (Koken, Tokyo, Japan) in phosphate-buffered saline (PBS: 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, at pH 7.40). Cells were incubated in Eagle's minimum essential medium (MEM: M4655, Sigma, St. Louis, Missouri) supplemented with 10% horse serum (Gibco, Invitrogen, Carlsbad, California) and 4% chicken embryo extract, at 37 ºC and in 5% CO_2. Chicken embryo extract was prepared by the following procedure.: Thirteen-day-old chicken embryo bodies were blended in the same volume (milliliters) of PBS to total weight (grams) at 4 ºC, and the supernatant collected by centrifugation at 9000 × g for 1 h was used as the chicken embryo extract. Cell fusion was initiated 2 or 3 days after sowing, and the culture medium was replaced with fresh medium supplemented with 1 μM Cytosine-D-arabinofuranoside hydrochloride (Ara-C, an inhibitor of DNA polymerases; C6645; Sigma) and 5 mM potassium chloride. Ara-C inhibited the proliferation of mononucleated cells. Potassium inhibited autonomous contractions induced subsequently in myotubes.[5] The myotubes

matured to striated cells by the fifth day after sowing, and the cultures were used for experiments.

Experimental procedures. Thirty minutes before the trials, the culture medium was replaced with fresh medium with or without an inhibitor [wortmannin (100 nM, inhibitor of PI3K; W1628; Sigma), rapamycin (10 ng/mL, inhibitor of TOR; R0395; Sigma), or U0126 (10 μM, inhibitor of MEK; U120; Sigma)]. Cells were then subjected to uniaxial cyclic stretching (110 % in the original length at 1/6 Hz) or were left non-stretched as a static control. Some of the cells were incubated statically with IGF-1 (10 ng/mL; #100-11; PeproTech Inc., Rocky Hill, New Jersey) as a positive control with the activated PI3K/Akt/TOR pathway. All trials were conducted at 37 ºC with 5% CO_2. After 72-h incubation, myotube diameters were determined by the method described in what follows.

Application of mechanical stretching. The stretching of cells was carried out using a cyclic stretching apparatus described previously.[13] Briefly, cells were attached on a sheet of elastic silicon membrane (46 mm × 11 mm and 0.2 mm thick) in a glass chamber (inside base area 60 mm × 30 mm). One longitudinal end of the silicone

membrane was fixed in the chamber, and the other was connected to the attachment of the stretching machine. This attachment was moved in a simple harmonic manner following the rotation of the motor, and the silicone membrane was elongated periodically by this movement at 1/6 Hz to 110% along its longitudinal axis. Cells on the silicone membrane were subjected to cyclic stretch as the membrane was elongated and relaxed.[13] Cyclic stretch at the rate of 1/6 Hz was performed continuously for 72 h in hypertrophy experiments and 5-60 min in the assessment of Akt phosphorylation. In this study, myotubes were stretched approximately in the longitudinal direction, because they were laid along micro-grooves molded along the axis of stretching on the surface of the silicone membrane.

Determination of myotube diameters. After experimental trials, the cells were washed three times with ice-cold PBS and fixed in 4% paraformaldehyde in PBS at room temperature (RT) for 15 min. Differential interference contrast (DIC) images of the cells through a light microscope (Eclipse TE2000, with a 20 × objective lens; Nikon, Tokyo, Japan) were monitored with a CCD camera system (Penguin 600CL; Pixera, San Jose, California) and stored on the hard disk of a personal computer. The processing of image files and determination of myotube diameters were accomplished using Image

software (Scion, Frederick, Maryland). To obtain randomized determination, the following procedures were performed. In short, one image was taken randomly in each area that divided a sheet of silicon membrane evenly into nine parts (three parts in length and width); in each image, myotubes crossing one diagonal were selected; at the crossing points, myotube diameters were gauged.

Western blot analysis. Experimental trials for this analysis were performed under serum-starved conditions to obtain sufficient detection to evaluate the difference in Akt phosphorylation between experimental groups. Cells were serum starved for 6 h before trials. After experimental trials, the cells were washed twice with ice-cold PBS and lysed in 2× sodium dodecylsulfate (SDS) sample buffer [62 mM Tris-HCl (pH 6.8), 4% SDS, 10% mercaptoethanol, 0.02% bromophenol blue, 20% glycerol]. The lysates were briefly sonicated, boiled, and spun down. The protein concentrations of the supernatants were measured by a modified Bradford technique.[22] Total protein of 20-50 μg was fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to an Immobilon P membrane (Millipore, Billerica, Massachusetts). The membrane was blocked for 1 h with PBS containing 5% nonfat dry milk and 0.1% Tween 20. Total Akt or phospho-Akt (Ser473) was detected on a duplicate membrane

using rabbit polyclonal antibodies (1:1000 dilution; 9272 and 9271; Cell Signaling Technology, Beverly, Massachusetts). The membrane was developed using alkaline phosphatase-conjugated secondary antibodies and an alkaline phosphatase conjugate substrate kit following manufacturer's instructions (170-6518 and 170-6432; Bio-Rad Laboratories, Hercules, California).

Statistical analysis. Results are presented as mean ± standard error of the mean (SEM). Statistical significance was calculated using analysis of variance followed by Tukey's post- hoc analysis. $P < 0.05$ was considered statistically significant.

Results

Myotube hypertrophy induced by cyclic stretching. We first examined the influence of cyclic stretching (110 % in the original length at 1/6 Hz) on myotube thicknesses. After 72-h stretching, highly thickened myotubes were observed in stretched cultures, but not in static cultures (Fig. 1A and B, dagger in Fig. 1D). In stretched cultures, the average myotube diameter was 30 ± 1.91 μm ($N = 91$), which is significantly larger than the diameter (20 ± 0.66 μm, $N = 107$) in static cultures ($P < 0.01$; Fig. 1C and D). This indicates clearly that myotubes were hypertrophied by cyclic

stretching.

Involvement of PI3K/Akt/TOR pathway in stretch-induced myotube hypertrophy. To examine the involvement of the PI3K/Akt/TOR signaling pathway in stretch-induced myotube hypertrophy, we carried out pharmacologic experiments using inhibitors that would interfere with this pathway. IGF-1 stimulation that activates the pathway was used as a positive control. The PI3K inhibitor, wortmannin, decreased the diameter of stretched- myotubes by 25% ($P < 0.01$; Fig. 2A, STR), as well as that of positive controls by approximately 30% ($P < 0.01$; Fig. 2A, IGF-1). Meanwhile, the drug showed no effect on static myotubes (Fig. 2A, NON). Although wortmannin suppressed both stretch- and IGF-1-induced hypertrophies, there was a smaller effect on stretch-induced hypertrophy (Fig. 2A, STR hatched). The TOR inhibitor rapamycin behaved similarly to wortmannin, but it suppressed the hypertrophy of stretched myotubes more severely (Fig. 2B, STR crosshatched). These results indicate that the PI3K/Akt/TOR pathway plays an important role in stretch-induced myotube hypertrophy. It also suggests involvement of other mechanisms, because the degree of suppression by these inhibitors in stretched myotubes was smaller compared with that in positive control (IGF-1-stimulated myotubes).

Effect of inhibition of MEK/ERK pathway on stretch-induced myotube hypertrophy. To examine the involvement of the MEK/ERK pathway in stretch-induced myotube hypertrophy, the MEK inhibitor, U0126, was employed. Unexpectedly, U0126 showed little effect on stretch induced hypertrophy (Fig. 2C; the diameter of cyclically stretched myotubes in the absence or presence of 10 μM U0126 increased 1.14- and 1.16-fold, respectively, relative to each unstretched control myotube); and furthermore, U0126 increased myotube diameters significantly in both stretched and static cultures ($P < 0.01$; Fig. 2C, STR or NON). This result suggests that the MEK/ERK pathway is involved instead in the basal down-regulation of myotube thickness.

Akt phosphorylation induced by cyclic stretching. Following the aforementioned indication of involvement of the PI3K/Akt/TOR pathway in stretch-induced myotube hypertrophy, we investigated whether Akt phosphorylation is promoted by cyclic stretching. First, time-course analysis was performed by western blotting, which showed that cyclic stretching for 5 or 60 min elevated the phosphorylation levels of Akt significantly ($P < 0.05$), as did IGF-1 stimulation for the positive control ($P < 0.01$; Fig.

3A). Further investigation showed that elevated phosphorylation induced by stretching for 5 min was significantly reduced by wortmannin beyond the non-stretched level ($P < 0.01$; Fig. 3B); essentially the same results were obtained in the samples stretched for 60 min (data not shown). These data suggest that cyclic stretching promotes Akt phosphorylation *via* PI3K, as observed in the case of IGF-1 stimulation.[25]

Discussion

The PI3K/Akt/TOR pathway is thought to work on the acceleration of protein synthesis[6,14,25] and in suppression of protein degradation.[30] In skeletal muscle mechanical stimulation[10,11] or IGF-1 treatment[25] will activate the PI3K/Akt/TOR pathway to induce hypertrophy. Furthermore, activation of Akt is sufficient to prevent muscle atrophy[6], and the kinase activity of Akt is essential for IGF-1-induced hypertrophy.[25] These findings imply that the PI3K/Akt/TOR pathway plays a pivotal role in muscle hypertrophy; however, it is still unclear whether activation of the PI3K/Akt/TOR pathway is indispensable for mechanically induced muscle hypertrophy. We demonstrated that cyclic stretching-induced muscle hypertrophy (Fig. 1) is significantly suppressed by the inhibition of the PI3K/Akt/TOR pathway (Fig. 2A and B). This is the first evidence to demonstrate that the PI3K/Akt/TOR pathway plays a

crucial role in skeletal muscle hypertrophy induced by cyclic mechanical stimulation as in IGF-1-induced hypertrophy.[25]

Cyclic stretching increased the phosphorylation levels of Akt in a biphasic manner (Fig. 3A), similar to that observed in cycically stretched bovine aortic endothelial cells (BAECs).[32] Fast activation of Akt by mechanical stimulation has been shown in exercised rat muscles[8] and in cyclically stretched BAECs.[32] The first peak at 5 min after the onset of stretching seems to be too early to be mediated by *de-novo* synthesis of growth factors described in what follows.[24] In BAECs, an increase of $[Ca^{2+}]i$ was required for the early-phase Akt activation, suggesting that certain Ca^{2+}-mediated mechanisms are involved in the fast Akt activation in mechanically stimulated myotubes. The second peak that appeared 60 min after stretching was possibly mediated by the autocrine/paracrine loop of IGF-1, because the expression of IGF-1[25] has been known to be increased by a mechanical load.[2,12,14,23,29] Actually, S6 kinase phosphorylation, the downstream effector of the PI3K/Akt/TOR pathway, is known to be promoted by an autocrine mechanism in response to acute stretching of myotubes.[4] However, the mechanically induced activation of the PI3K/Akt/TOR pathway seems to be modulated by other mechanisms, based on the following observations. Functional IGF-1 receptors

are not necessary for Akt phosphorylation and load-induced hypertrophy in muscles.[29] Furthermore, TOR/S6 kinase is regulated by mechanical stresses in a PI3K/Akt-independent manner.[14,15] Further studies are required to determine the molecular mechanism(s) underlying the mechanically induced activation of the PI3K/Akt/TOR pathway.

On the other hand, it is likely that an unidentified molecular signal axis is involved in stretch-induced myotube hypertrophy, which may or may not work with the PI3K/Akt/TOR pathway. We initially speculated that the MEK/ERK pathway would be a possible unidentified mechanism[26], because some reports showed that the MEK/ERK pathway positively regulated differentiation of myoblasts[18,20,35], and cyclic stretching upregulated the activity of the MEK/ERK pathway in C2C12 myoblasts.[18] The MEK/ERK pathway does not seem to be involved in stretch-induced hypertrophy, because inhibition of the MEK showed a small effect on the stretch-induced hypertrophy (Fig. 2C). A possible interpretation is that mechanically activated Akt inhibited the mechanically induced MEK/ERK activation in differentiated chick primary myotubes, based on the fact that constitutively active Akt inhibits serum-induced MEK/ERK activation.[26] Furthermore, inhibition of the MEK/ERK

pathway increased myotube diameters both in static and stretched cultures (Fig. 2C). Earlier studies showed that the spontaneous activity of the Raf-MEK-ERK pathway is downregulated in differentiated myotubes[18], and downregulation of Raf-MEK-ERK signaling induces myotube hypertrophy in differentiated myotubes through an upregulation of transcription levels of myogenin.[26] Our results of MEK inhibition are consistent with these results. This implies that MEK/ERK is not essential for mechanically induced myotube hypertrophy and potentially downregulates myotube thickness in the post-differentiation stage.

In conclusion, cyclic stretching promotes hypertrophy in cultured skeletal myotubes primarily through activating the PI3K/Akt/TOR pathway, although the underlying mechanisms of stretching activation of this pathway and the contribution of other molecular signaling pathways remain to be solved. Meanwhile, the MEK/ERK pathway is implicated minimally in stretch-induced hypertrophy, but it participates in down-regulation of spontaneous hypertrophy in post-maturation myotubes.

Abbreviations

Ara-C, cytosine-D-arabinofuranoside hydrochloride

DIC, differential interference contrast

ERK, extracellular signal-regulated kinase

IGF-1, insulin-like growth factor-1

MAPK, mitogen-activated protein kinase

MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase

MEM, Eagle's minimum essential medium

PBS, phosphate-buffered saline

PI3K, phosphatidylinositol 3-kinase

RT, room temperature

SDS, sodium dodecylsulfate

SDS-PAGE, sodium dodecylsulfate- polyacrylamide gel electrophoresis

TOR, target of rapamycin

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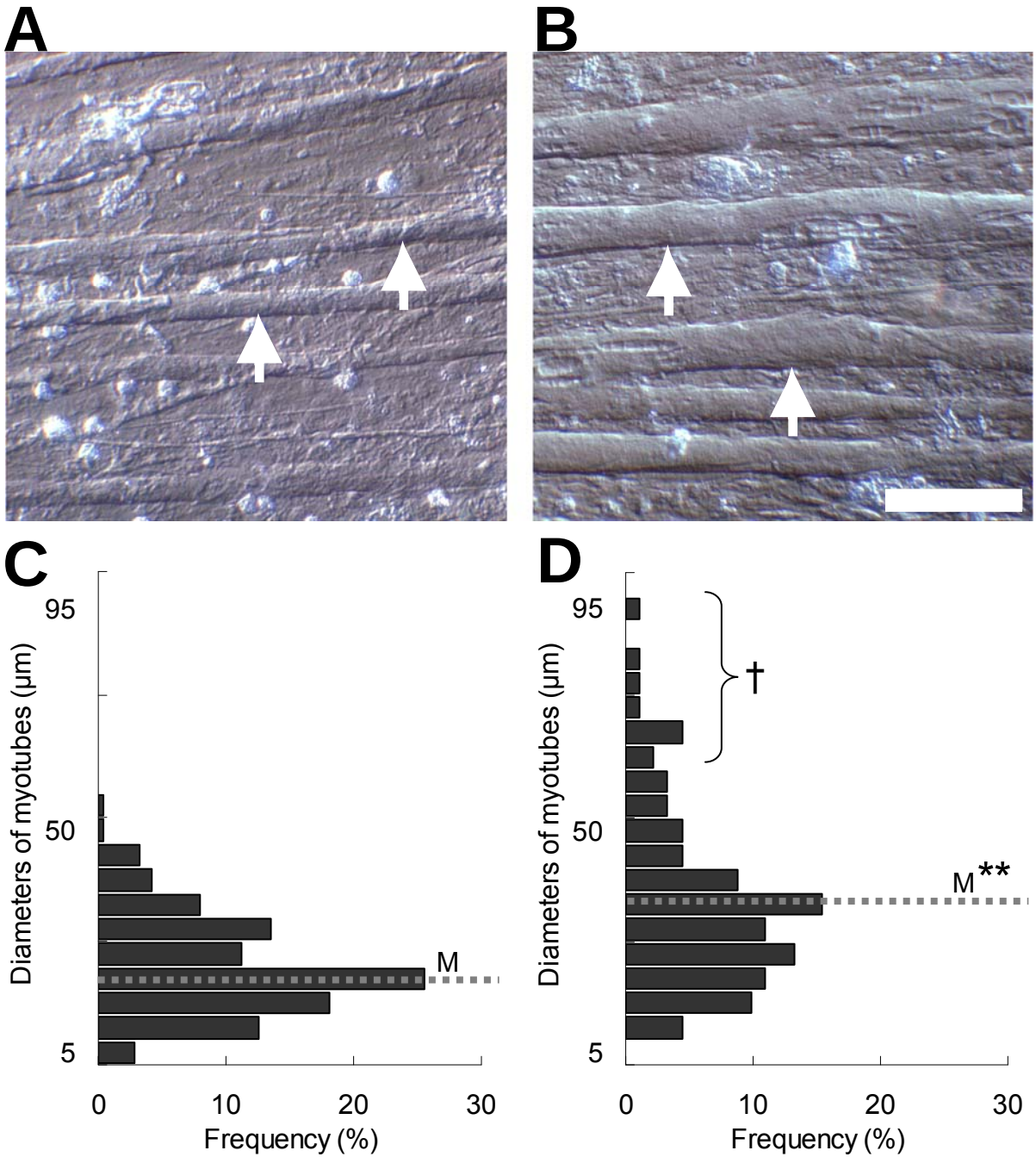
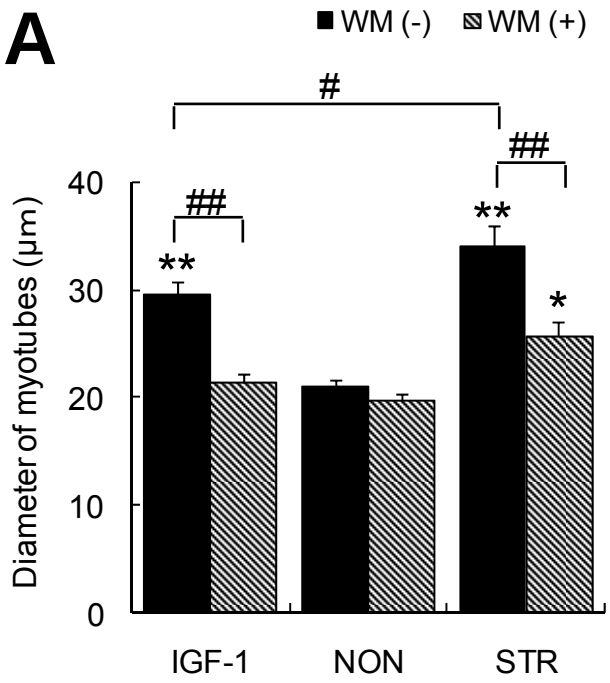

A
B
C
D
95
50
5
Diameters of myotubes (μm)
M
0
10
20
30
Frequency (%)
†
M**

Figure 1. Stretch-induced hypertrophy in cultured skeletal myotubes.

Differential interference contrast microscopic (DIC) images of non-stretched (A) and stretched myotubes (B). Myotubes are indicated by arrows. Scale bar is 100 μm. (C, D) Histograms of myotube diameters derived from DIC images: (C) n =107 and (D) n = 91. (A) and (C) were cultured statically, and (B) and (D) underwent cyclic stretching. Stretched myotubes in (B) were thicker than static myotubes in (A). Extremely thick myotubes (dagger) were observed in stretched culture (D), but not in static culture (C). The mean of myotube diameters (M) was significantly enlarged in stretched culture as shown in (D) (**$P < 0.01$).


A
WM (-)
WM (+)
#
##
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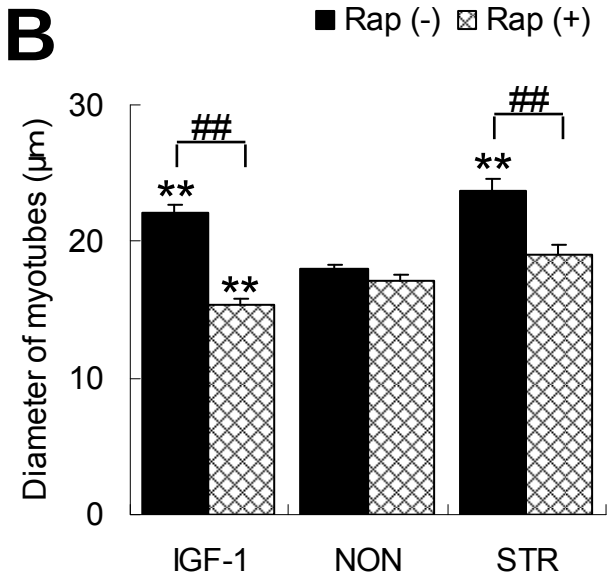
*
40
30
20
10
0
Diameter of myotubes (μm)
IGF-1
NON
STR


B
Rap (-)
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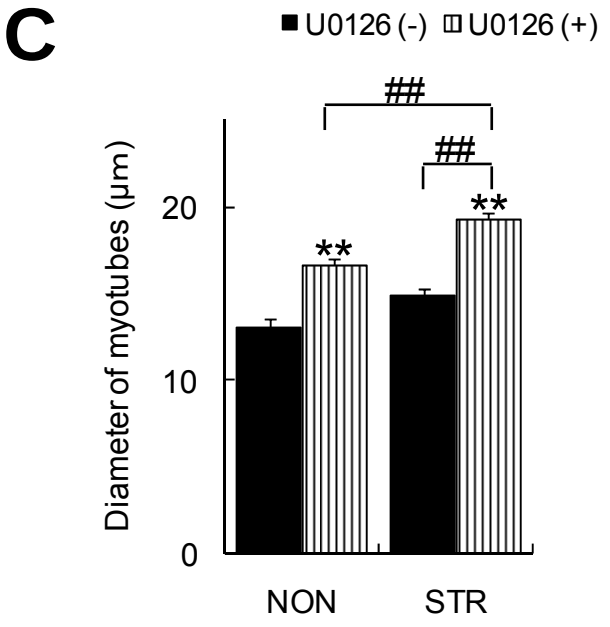
Diameter of myotubes (μm)
IGF-1
NON
STR


C
U0126 (-)
U0126 (+)
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##
**
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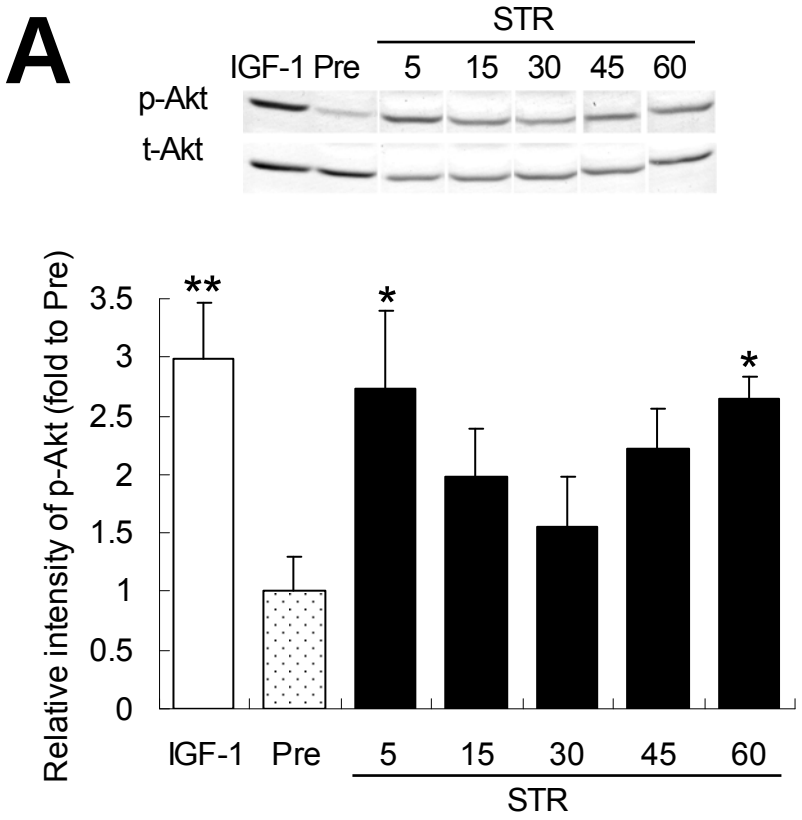
20
10
0
Diameter of myotubes (μm)
NON
STR

Figure 2. Effects of inhibitors on stretch-induced hypertrophy.

The effect of inhibition on myotube diameter is shown. Myotubes were treated for 72 h with: 100 nM wortmannin, an inhibitor of PI3K (A); 10 ng/ml rapamycin, an inhibitor of TOR (B) ; or 10 μM U0126, an inhibitor of MEK (C). The vertical axes indicate relative myotube diameter normalized to the mean diameter of non-stretched myotubes (error bars: SEM). Solid bars represent myotubes without inhibitors, and others are myotubes with inhibitors, respectively. NON, myotubes cultured statically; STR, stretched; IGF-1, IGF-1 stimulation. Statistical significance: $*P < 0.05$, $**P < 0.01$ vs. NON solid; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. PI3K and TOR inhibitors affected IGF-1 and stretch-induced myotube hypertrophy, but MEK/ERK inhibitor did not.


A
STR
IGF-1 Pre 5 15 30 45 60
p-Akt
t-Akt
Relative intensity of p-Akt (fold to Pre)
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2.5
2
1.5
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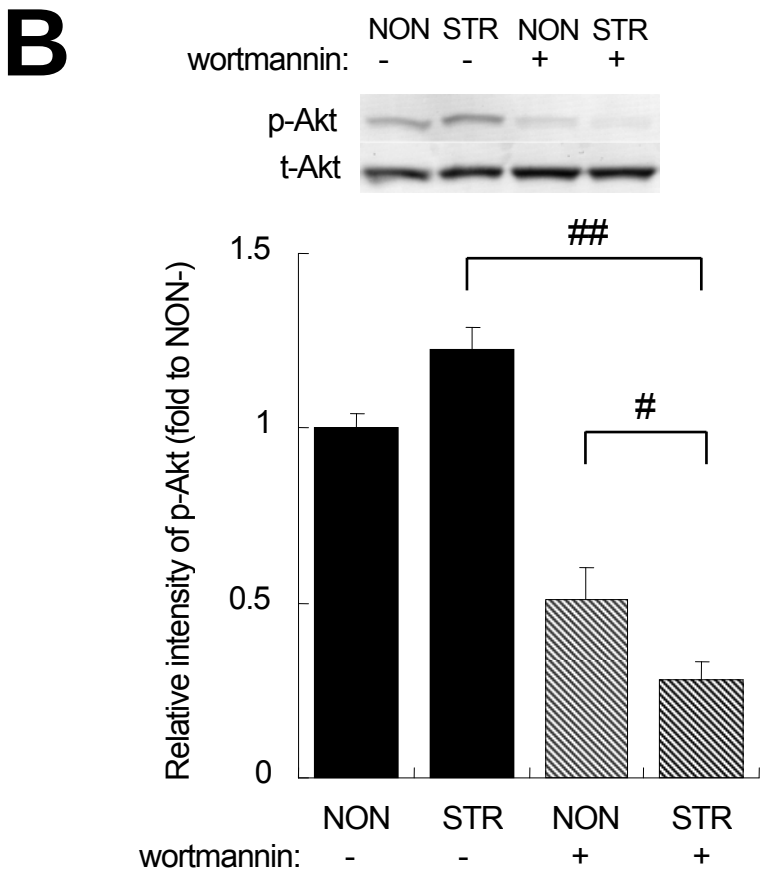
IGF-1 Pre 5 15 30 45 60
STR


B
NON STR NON STR
wortmannin: - - + +
p-Akt
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Relative intensity of p-Akt (fold to NON-)
1.5
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0.5
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NON STR NON STR
wortmannin: - - + +

Figure 3. Phosphorylation of Akt induced by cyclic stretching.

(A) Upper panel shows a representative result of immunoblot analysis showing total (t-Akt) and phospho-Akt (p-Akt) levels in the myotubes treated with 10 ng/ml IGF-1 for 60 min, or cyclic stretching (110 %, 1/6 Hz) for 5-60 min. (B) Akt phosphorylation level at 5 min in wortmanin-treated myotubes. The results of the densitometoric analyses of the immunoblot membranes [upper panels in (A) and (B)] are depicted in the lower panels as the ratio of p-Akt against the t-Akt signal (mean ± SEM, $n = 3$), respectively. Vertical axis implies relative p-Akt revel compared with pre-treated myotubes (A), or non treated myotubes (B). Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$; or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

和文抄録

Involvement of PI3K/Akt/TOR pathway in stretch-induced hypertrophy of myotubes

（伸張刺激により引き起こされる筋管細胞肥大への PI3K/Akt/TOR 経路の関与）

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

笹井　宣昌（指導：　河上　敬介　准教授）

骨格筋細胞は、機械ストレスにより肥大する。しかしながら、その分子メカニズムは十分に解明されてない。筋肥大に関与すると考えられている細胞シグナル経路として、 phosphatidylinositol 3-kinase (PI3K)/Akt/target of rapamycin (TOR) 経路や extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) 経路がある。本研究では、それら経路が、筋細胞の伸張刺激による肥大に関連するかどうかという点について調べた。トリ胸筋の初代培養による成熟した筋管細胞に、その長軸方向の周期的伸張（伸張率 10%, 1/6 Hz, 72 時間）を加えたところ、伸張しない細胞に比べ太さが約 1.6 倍に肥大した ($p < 0.05$)。この伸張刺激による肥大は、 PI3K の阻害剤 (wortrmannin) や、TOR の阻害剤 (rapamycin) により抑制された。さらに伸張刺激を 5 及び 60 分加えた細胞のリン酸化 Akt の割合は、刺激前に比べ凡そ 2.5 倍高かった ($p < 0.05$)。このシグナル亢進は wortrmannin により抑制された。一方、 MEK の阻害剤 (U0126) により、伸張刺激に関わらず細胞は肥大した。伸張刺激による肥大は殆ど変わらなかった。これらの結果から、伸張刺激による筋管細胞肥大に対し PI3K/Akt/TOR 経路が関連することや、 ERK の関連は僅かであることが示唆された。さらに ERK が、成熟した筋管の太さ制御に負に関わることも示唆された。