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POTENTIAL ROLE OF PHOSPHORYLATION OF p70^{S6K} IN DIFFERENT EXTENTS OF MUSCLE ATROPHY ACCORDING TO TYPE OF SKELETAL MUSCLE DURING ANKLE IMMOBILIZATION IN RAT

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ABSTRACT: Akt and p70 ribosomal S6 kinase (p70^{s6k}) play a critical role in regulation of skeletal muscle mass in response to a variety of mechanical stimuli. However, the regulation of these molecules under decreased muscle usage has rarely been investigated according to skeletal muscle types that affect the rate of disuse muscle atrophy. This study was conducted in rats to elucidate the responses of Akt and p70^{s6k} phosphorylation to ankle immobilization in different types of plantar flexors and its association with the muscle atrophy rate in the muscles. The results revealed that the rate of muscle atrophy in response to ten days of ankle immobilization was significantly less in the plantaris (PL) and gastrocnemius, which could not be completely immobilized with the ankle fixation, than in the soleus (SOL) ($P<0.05$). In accordance with these findings, the decrease in phosphorylation of p70^{s6k} (T389) that was observed in SOL ($P<0.001$) was not observed in PL, and its level even increased in fast-twitch white gastrocnemius (WG) ($P<0.05$). However, phosphorylation of Akt (S473) was lower in all three muscles in response to immobilization treatment. The Akt-independent phosphorylations of p70^{s6k} in PL and WG were further confirmed by the decreased phosphorylation of Akt (T308) and GSK3α. These results suggest that the skeletal muscle type-specific different rates of muscle atrophy that occur during ankle immobilization may be mediated by p70^{s6k}, which responds differently in the distinct types of skeletal muscle.

KEY WORDS: ankle immobilization, Akt, p70 ribosomal S6 kinase, skeletal muscle type

INTRODUCTION

Skeletal muscle, which is a largely plastic tissue, has a unique ability to convert extracellular mechanical information into molecular events that regulate its mass. Accordingly, if the mechanical input is reduced, muscle mass will decrease and vice versa. Because maintenance of the skeletal muscle mass makes a significant contribution to prevention of diseases and promotion of quality of life in modern society [20,21], characterizing molecular targets that are considered to be sensitive to mechanical stimuli has become very important for the development of appropriate therapy to prevent skeletal muscle loss. To date, the most widely spotlighted mechanism for regulating muscle mass has been Akt (also called protein kinase B) signalling [8,18]. This is because, in response to mechanical stimuli, Akt can affect a variety of growth regulatory molecules such as glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin complex 1 (mTORC1), and forkhead box O (FoxO), governing both protein synthesis and protein degradation, of which balance is a major determinant of skeletal muscle mass [2,3,25].

The regulation of protein synthesis and muscle growth by mTORC1 is primarily mediated by its ability to phosphorylate p70 ribosomal S6 kinase (p70^{s6k}) directly at Thr-389, a critical site for its kinase activity. Phosphorylation of the ribosomal S6 subunit protein by activated p70^{s6k} enhances translational efficiency in the stage of translation initiation by stimulating selective translation of mRNAs with the 5' oligopyrimidine tract, which generally encode proteins involved in the translational apparatus [6,17]. Thus, phosphorylation of p70^{s6k} as a typical readout of mTORC1 signalling has also been intensively studied to enable interpretation of the effects of mechanical stimuli on protein synthesis and muscle growth, as well as activation of Akt.

As mentioned above, skeletal muscle mass decreases with reduced muscle use, and many studies have demonstrated that down-regulations of the two mechano-sensitive molecules, Akt and p70^{s6k}, are the major underlying mechanisms of disuse muscle atrophy [10,27]. However, despite the fact that the severity of disuse muscle atrophy differs according to skeletal muscle type, which is characterized by several factors including the number of joints that the muscle crosses and the muscle fibre type, few studies have been conducted to determine whether and how these two

molecules are involved in the varying degree of muscle atrophy according to skeletal muscle type. Therefore, identifying different actions of Akt and $p70^{s6k}$ based on the types of skeletal muscles under a muscle disuse condition would help clarify the properties of those molecules that mediate between the variations in disuse muscle atrophy and the factors responsible for such atrophy.

This study was conducted to determine how Akt and $p70^{s6k}$ are related to the difference in the rate of disuse muscle atrophy according to different types of muscle. To accomplish this goal, we applied ankle immobilization of the rat hindlimb in a full plantar flexion, and then analysed three different plantar flexor muscles (soleus, SOL; plantaris, PL; gastrocnemius, G) that would be subjected to reduced muscle usage by the treatment, but would have different atrophy conditions according to their distinct muscle characteristics (i.e. oxidative/slow-twitch SOL that crosses only one joint, and glycolytic/fast-twitch PL and G [white] that cross two joints).

MATERIALS AND METHODS

Animals. The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of the Laboratory of Animal Resources at Seoul National University for the ethical use of animals in research. Nine-week-old male Sprague-Dawley rats weighing 315-335 g (Orient Bio Ltd., Seoul, Korea) were sustained in individual mesh cages in a room maintained at approximately 23ºC under a 12 h:12 h light:dark cycle. Rats were provided with a slightly modified AIN-93M diet and water *ad libitum* throughout the experimental period.

Immobilization procedure

After three days of acclimation, animals were anaesthetized by an intramuscular injection of Zoletil (10 mg/kg) and acepromazine (0.5 mg/kg) into their inner thigh and then subjected to unilateral hindlimb immobilization by attaching a pre-made immobilization device as described in our previous study [26]. The immobilized legs were maintained with a complete plantar-flexion of the ankle joint, but not the knee joint (Fig. 1). The animals were checked daily to ensure complete ankle immobilization and detect any movements of other joints, and adjustments were made immediately as necessary. In a pilot study (n=4 in each weight- and age-matched group), we compared the mass of muscles (PL and gastrocnemius) from the contralateral non-immobilized legs of animals subjected to the immobilization device for ten days (group 1) with that of either the left or right legs of animals that were not dressed in the immobilization device (group 2). A similar comparison was also performed in terms of the phosphorylation of Akt. The results revealed that there were no statistical differences in the muscle mass of the two groups (PL: group 1, 106.54 ± 9.69; group 2, 104.39 ± 7.31 and 102.41 ± 5.31, respectively; Gastrocnemius: group 1, 529.76 ± 32.4; group 2, 524.83 ± 38.14 and 513.14 ± 21, respectively; mg/100g body weight) or in the phosphorylation of Akt (data not shown), demonstrating the validity of using muscles from the contralateral non-immobilized leg as an internal control in this study.

Tissue preparation

Following ten days of immobilization, animals were sacrificed by exsanguination while ensuring no weight bearing load was placed on the immobilized leg after anaesthesia as described above. At the time of sacrifice, the SOL, PL, and G muscles were excised from the animals in a fed state while removing any connective tissue, after which they were weighed, frozen in liquid nitrogen, and stored at -80ºC for later analysis.

Protein extraction

A portion of muscles was homogenized with Polytron in an ice-cold lysis buffer containing 50 mM HEPES-NaOH (pH 7.5), 150 mM sodium chloride, 2 mM EDTA (pH 8.0), 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium vanadate, 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail for 10 s three times each. The homogenate was incubated on ice for 30 min, after which it was centrifuged at 15000 g for 30 min to separate the insoluble pellet. An aliquot of the supernatant was collected for analysis, and the protein concentration was determined in triplicate by using

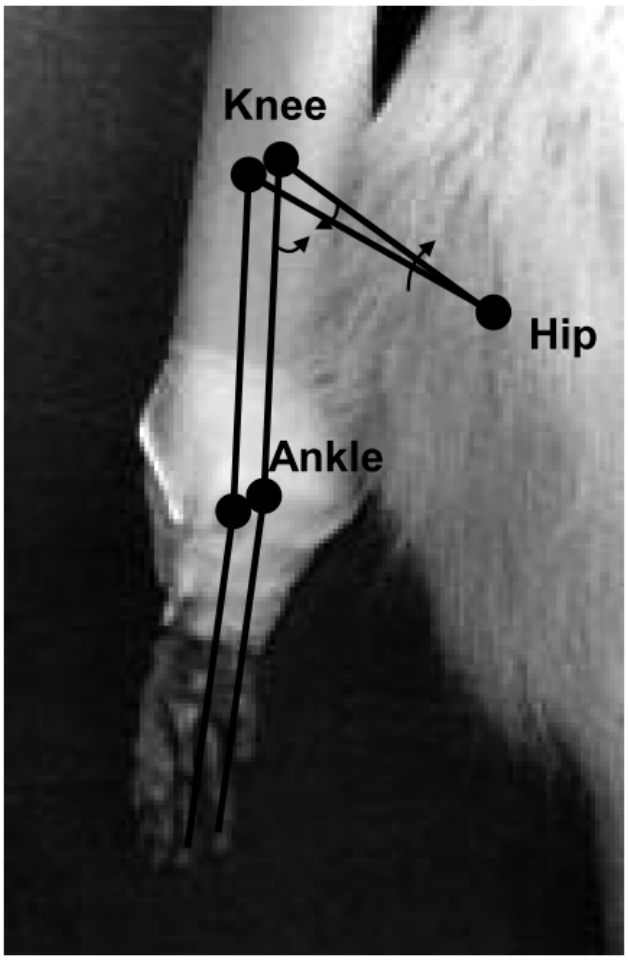

FIG. 1. DETECTED MOVEMENTS OF EACH JOINT UNDER THE ANKLE IMMOBILIZATION TREATMENT

a bicinchoninic acid protein assay according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL).

Western blot analysis

Samples were diluted with lysis buffer and sample buffer containing 120 mM Tris-HCl (pH 6.8), 4% w/v SDS, 20% glycerol, 0.025% w/v bromophenol blue, and 10% β-mercaptoethanol, after which they were boiled for 5 min. Equal amounts of the denatured protein samples were then resolved by SDS-PAGE on 10% acrylamide gels and subsequently transferred to a PVDF membrane (Millipore, Charlottesville, VA). The membranes and gels were all stained with Ponceau S and Coomassie Blue, respectively, to verify equal loading in all lanes. Next, blocking of the membrane was performed by incubation in 5% powdered milk in TBS-T (Tris-buffered saline, 1% Tween 20) for 1 h. After a brief wash, the membranes were probed with primary antibodies overnight at 4ºC and then washed with TBS-T for a minimum of 30 min. The membranes were then again probed with horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ) for 1 h at room temperature, followed by washing with TBS-T for a minimum of 30 min. After an additional incubation of the membrane with ECL plus (Pierce, Rockford, IL), bands from the membranes were visualized by exposure to Kodak X-ray film and quantified using the Quantity one 4.3.1. densitometric software (Bio-Rad, Hercules, CA).

Primary antibodies

The following primary antibodies were used: rabbit polyclonal phospho-Akt (Ser-473), phospho-Akt (Thr-308), total-Akt, phospho-$p70^{s6k}$ (Thr-389), total-$p70^{s6k}$ and rabbit monoclonal phosphor-GSK 3α from Cell Signaling Technology (Danvers, MA); mouse monoclonal GSK3 from Millipore (Charlottesville, VA).

Data analysis

Data analysis was conducted using SPSS 17.0. A paired *t* test was employed to identify differences between muscles from immobilized legs and muscles from the respective contralateral non-treated leg in each group (Δ). Comparisons among the changes (Δ) were assessed using one-way ANOVA, followed by Duncan's post hoc test. Contralateral muscles served as an internal control. All values are expressed as the mean ± standard error of the mean. Statistical significance was determined at $P<0.05$.

TABLE I. EFFECTS OF ANKLE IMMOBILIZATION ON PLANTAR FLEXOR MUSCLE MASSES (mg/100g BODY MASS)

	Soleus	Plantaris	Gastrocnemius
Contralateral	49.81±2.72	117.09±2.39	587.64±9.96
Immobilized	21.74±0.85*	64.95±2.75*	321.09±14.98*
% Δ[1]	-55.97±2.31	-44.56±1.82#	-46.71±3.18#

Values are expressed as the mean ±SE, n=6.
[1] Percentage change of the immobilized muscles when compared to their contralateral muscles.
Statistical significance: * P<0.001 vs. contralateral; # P<0.05 vs. SOL

RESULTS

Figure 1 shows that our hindlimb immobilization treatment was primarily aimed at complete fixation of the ankle joint in a plantar flexion position, resulting in no movement at SOL, which crosses only the ankle joint. Conversely, the immobilization allowed some movement at the knee (as well as the hip) joint, possibly allowing minor force generation at other plantar flexors such as PL and G muscles, which cross the knee joint as well as the ankle joint. The extent of muscle loss in those plantar flexors after 10 days of ankle immobilization revealed that PL and G muscles were less atrophied than SOL by 10% ($P<0.05$), although decreases in the masses were all significant in the three muscles ($P<0.001$) (Table 1).

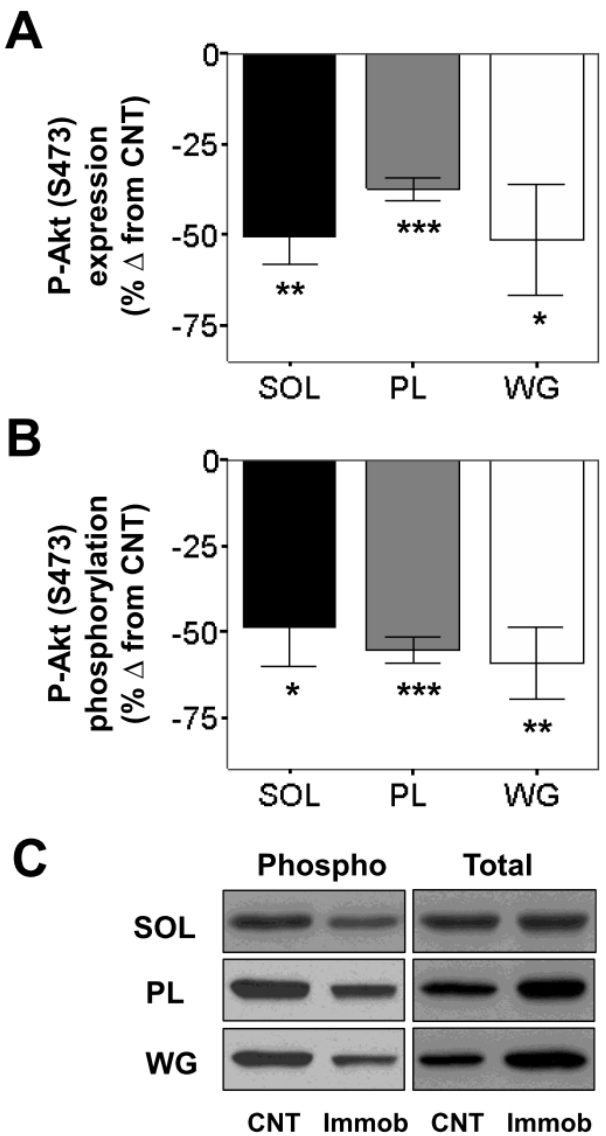

FIG. 2. DOWN-REGULATION OF AKT PHOSPHORYLATION IN PLANTAR FLEXORS BY ANKLE IMMOBILIZATION. EXPRESSIONS OF PHOSPHORYLATED AKT (S473) (A) AND PHOSPHORYLATIONS OF AKT (S473) (B) IN IMMOBILIZED PLANTAR FLEXORS (IMMOB) (SOL, SOLEUS; PL, PLANTARIS; WG, WHITE GASTROCNEMIUS) ARE REPRESENTED AS PERCENTAGE CHANGE (Δ) FROM THEIR RESPECTIVE CONTRALATERAL MUSCLES (CNT). (C) REPRESENTATIVE WESTERN BLOT BANDS ARE SHOWN. VALUES ARE EXPRESSED AS THE MEAN ±SE, N=6. STATISTICAL SIGNIFICANCE: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ VS. CNT

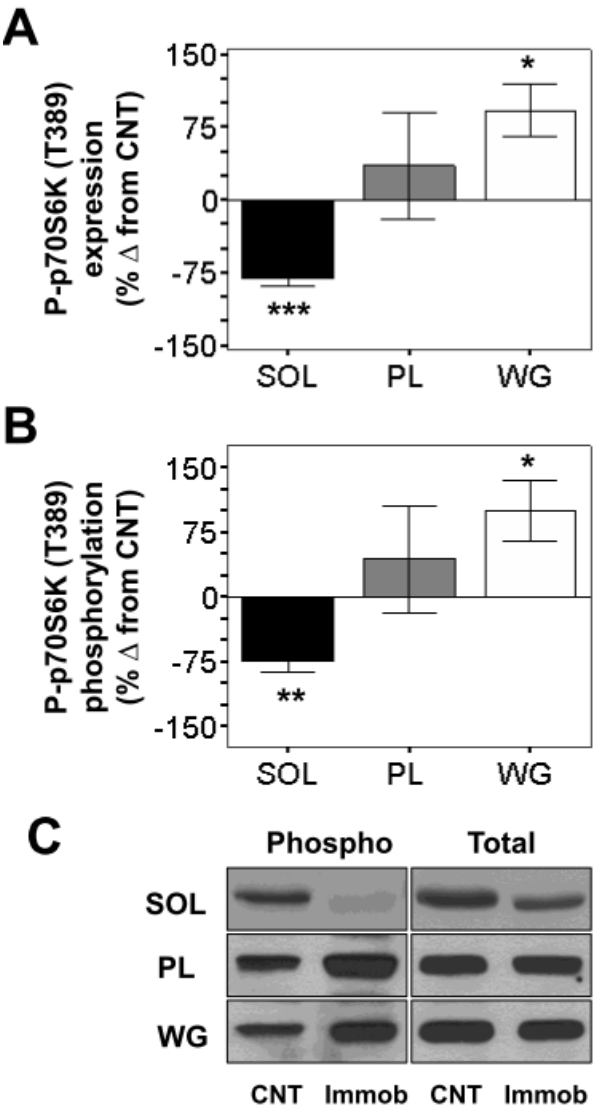

FIG. 3. DIFFERENTIAL $p70^{S6K}$ PHOSPHORYLATION IN PLANTAR FLEXORS BY ANKLE IMMOBILIZATION. EXPRESSIONS OF PHOSPHORYLATED $P70^{S6K}$ (T389) (A) AND PHOSPHORYLATIONS OF $p70^{S6K}$ (T389) (B) IN IMMOBILIZED PLANTAR FLEXORS (IMMOB) (SOL, SOLEUS; PL, PLANTARIS; WG, WHITE GASTROCNEMIUS) ARE REPRESENTED AS PERCENTAGE CHANGES (Δ) FROM THEIR RESPECTIVE CONTRALATERAL MUSCLES (CNT). (C) REPRESENTATIVE WESTERN BLOT BANDS ARE SHOWN. VALUES ARE EXPRESSED AS THE MEAN ± SE, N=6. STATISTICAL SIGNIFICANCE: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ VS. CNT

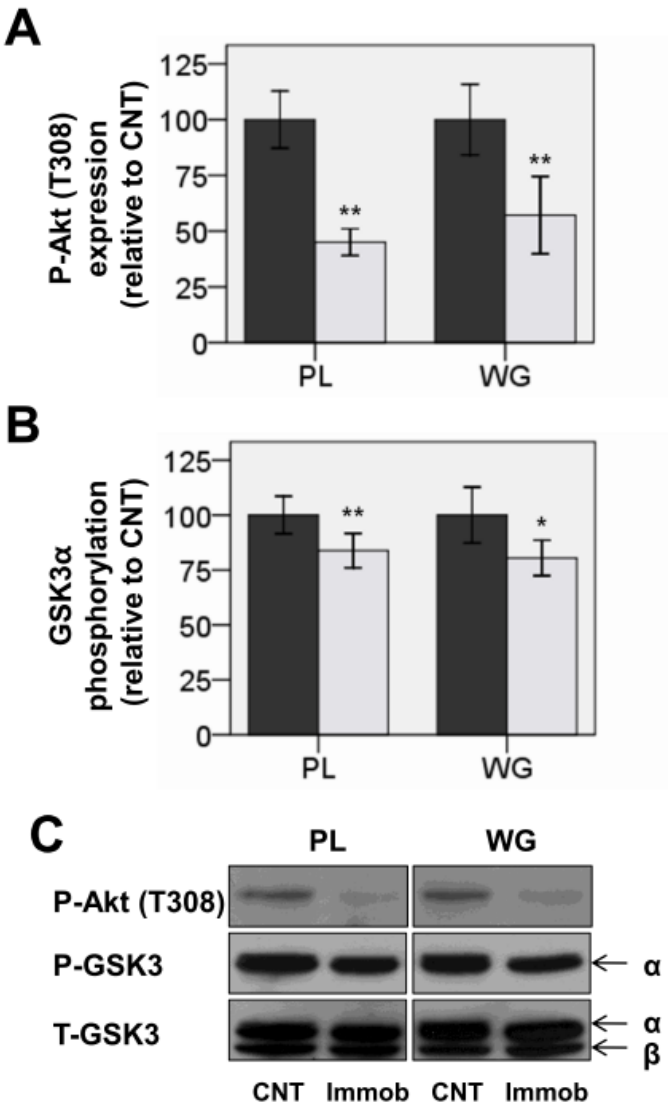

FIG. 4. CONFIRMATION OF DOWN-REGULATION OF AKT ACTIVATION IN PLANTARIS (PL) AND WHITE GASTROCNEMIUS (WG) BY ANKLE IMMOBILIZATION. EXPRESSIONS OF PHOSPHORYLATED AKT (T308) (A) AND PHOSPHORYLATIONS OF GSK3α (B) IN IMMOBILIZED PL AND WG (IMMOB, OPEN BARS) ARE REPRESENTED AS THE PERCENTAGE RELATIVE TO THEIR RESPECTIVE CONTRALATERAL MUSCLES (CNT, FILLED BARS). (C) REPRESENTATIVE WESTERN BLOT BANDS ARE SHOWN. VALUES ARE EXPRESSED AS THE MEAN ± SE, N=6 STATISTICAL SIGNIFICANCE: * $P<0.05$, ** $P<0.01$ VS. CNT

The phosphorylation status of Akt (Ser-473) and its downstream target, $p70^{s6k}$ (Thr-389), was assessed in SOL, PL, and white gastrocnemius (WG), which would have a greater fast-type portion than PL and red gastrocnemius. The results revealed that the levels of phosphorylated Akt and phosphorylation of Akt at Serine 473 were significantly decreased by ankle immobilization in all three plantar flexors (Fig. 2), while, in PL and WG, the expressions of total Akt were increased ($P<0.05$, data not shown). Levels of phosphorylated $p70^{s6k}$ and phosphorylation of $p70^{s6k}$ at Thr-389 were also reduced in SOL by the immobilization. However, in PL, these levels were not changed, and they even increased in WG with the same immobilization treatment, indicating divergent $p70^{s6k}$ signalling from the activation (phosphorylation) of Akt in these muscles (Fig. 3). To identify any possible activation of Akt that may contribute to the observed $p70^{s6k}$ signalling in PL and WG, we also assessed the phosphorylated states of Akt at Thr-308, another critical residue for the activation of Akt, and phosphorylation of GSK3α at Ser-21, a direct phosphorylation site of Akt, in PL and WG. The results confirmed that Akt was deactivated by the ankle immobilization in the PL and WG in that the values all significantly decreased in the muscles (Fig. 4).

DISCUSSION

Although Akt and $p70^{s6k}$ have largely been acknowledged as critical molecules that interpret extracellular mechanical information to

regulate skeletal muscle mass, their regulation by types of skeletal muscles in certain muscle disuse conditions has hardly been studied yet. In this study, we found that phosphorylation of $p70^{s6k}$ by ankle immobilization was differentially regulated by types of skeletal muscle, and this was associated with different rates of muscle atrophy in the muscles.

Our immobilization treatment was sufficient to reduce phosphorylation of Akt in both PL and WG as well as in SOL, which has not been seen in other muscle disuse models such as hindlimb suspension, which did not reduce phosphorylation of Akt in the gastrocnemius [11]. However, we found that the phosphorylation of $p70^{s6k}$ was not altered in PL, and that it even increased in WG, despite the decreased activation of Akt. Furthermore, these divergent actions of $p70^{s6k}$ were related to the lesser degree of atrophy in the PL and G muscles when compared to that of the SOL. These results suggest that activation of $p70^{s6k}$ may play an important role in different rates of atrophy in different types of skeletal muscle while deactivation of Akt may be necessary for the induction of muscle atrophy, at least more than $p70^{s6k}$, during immobilization.

Three factors are likely responsible for the observed differences in the rate of muscle atrophy and phosphorylation of $p70^{s6k}$ shown by different types of skeletal muscles: the number of joints that each muscle crosses, the length of muscle immobilized, and the muscle fibre type. Because only the ankle joint of the rat hindlimb was directly fixed under our immobilization treatment, PL and G muscles, which cross the knee joint as well as the ankle joint, could still be used as knee flexors, resulting in transient transition of mechanical force on those muscles. Thus, this subtle difference in mechanical input may have allowed mechanosensors in the muscles to induce skeletal muscle type-specific mechanotransduction (including $p70^{s6k}$), which could influence the rate of muscle atrophy, as previously demonstrated [7]. Second, muscle atrophy occurs in inverse proportion to the length of the immobilized muscle; in addition, muscle disuse in a stretched position can induce hypertrophy or activation of $p70^{s6k}$ [5,9,13]. However, this factor is hard to consider because our immobilization resulted in the ankle being maintained at full plantar flexion, which makes all of the plantar flexors, including the PL and G muscles, become shortened. Finally, it is generally accepted that fast-twitch muscles are less susceptible to disuse muscle atrophy. In accordance with this, the results of the present study revealed that only WG, the most fast-twitch muscle among the muscles employed in this study, underwent an increase in $p70^{s6k}$ phosphorylation, while the next fastest twitch muscle, PL, showed no decrease in the phosphorylation of $p70^{s6k}$, which was only observed in the slowest twitch muscle, SOL. Therefore, the results of this study indicate that $p70^{s6k}$ can be phosphorylated in response to the possible force generation according to the muscle fibre type, which may influence the rate of muscle atrophy during immobilization.

Although many studies have demonstrated that Akt and $p70^{s6k}$ are all activated in response to various types of mechanical stresses that lead to muscle growth [1,4,22,24], several studies have also shown that their regulation is not responsive to or dissociable from each other with altered (decreased or increased) mechanical stimuli [14,15,16,23]. In support of this, the present study showed increased phosphorylation of $p70^{s6k}$ in WG which was independently regulated from Akt under the immobilization treatment. This finding suggests that there is an undefined pathway(s) responsible for the Akt-independent activation of $p70^{s6k}$ (or mTORC1) which functions to alleviate severe disuse muscle atrophy. It has been reported that inhibition of phospholipase D (PLD)-mediated PA synthesis prevented mechanical stimuli-induced elevation of PA and the concomitant increase of $p70^{s6k}$ phosphorylation that is not inhibited by Akt inhibitor [12,19], indicating that one possible mechanism for the results presented here may involve an increase in phosphatidic acid (PA) via intermittent force generation. Future studies should be conducted to address this issue.

CONCLUSIONS

In conclusion, the present study demonstrated that, in immobilized plantar flexors, phosphorylation of $p70^{s6k}$, but not Akt, is differently regulated according to the types of skeletal muscle (i.e. no change and increase in PL and WG, respectively, which had some degree of movement, as opposed to SOL). Furthermore, this different regulation was associated with different rates of muscle atrophy, indicating that $p70^{s6k}$ is a potential mediator of the skeletal muscle type-specific different rates of muscle atrophy during immobilization treatment.

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