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Original Article

Decrease of Both Cofilin and LIM Kinase Phosphorylation in the Skeletal Muscles of Immobilization-induced Atrophy Rats

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Abstract. [Purpose] Immobilization-induced atrophy is a general phenomenon caused by prolonged muscle disuse associated with orthopaedic conditions. However, changes in the phosphorylation of atrophy-related cofilin and LIM kinases are still poorly understood. In this study, we examined whether or not phosphorylation of cofilin and LIM kinases is altered in the skeletal muscles of rats after 3, 7, 14, and 21 days of cast immobilization. [Methods] We used two-dimensional gel electrophoresis, mass spectrometry, and western blotting to examine protein expression and phosphorylation in atrophied rat gastrocnemius muscles. [Results] The expression of the cofilin was detected in gastrocnemius muscle strips using proteomic analysis. Cast immobilization after 3, 7, 14, and 21 days significantly diminished the phosphorylation of cofilin and LIM kinases. [Conclusion] The present results suggest that cast immobilization-induced atrophy may be in part related to changes in the phosphorylation of cofilin and LIM kinases in rat skeletal muscles.
Key words: Cofilin, LIM kinases, Skeletal muscle atrophy

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INTRODUCTION

Cofilin is an actin binding protein with a low molecular weight of about 19 kDa. It was first extracted and purified from embryonic chick brain extracts, and is ubiquitously expressed protein in eukaryotic cells, where it regulates actin filament dynamics and reorganization, and other functions for cellular viability[1–3)]. Actin filament dynamics and reorganization are fundamental cell activities, which include cell division, morphogenesis, migration, endocytosis, and gene expression[4–6)]. Cofilin binds to fibrous actin changing the fibrous actin to globular actin[2)]. This process requires the dephosphorylation of cofilin by phosphatases[7, 8)]. Phosphorylation abolishes cofilin activity and inhibits the severing function of cofilin[9, 10)], and LIM kinases, a serine kinase, phosphorylates cofilin[9, 11)]. A description of the membranes of the LIM kinase family of serine kinases, which include LIM kinase 1 and 2, has been published[9)]. Although the exact signaling pathway for the activation of LIM kinases is not fully understood, these proteins regulate actin polymerization via activation and inactivation of cofilin[9–11)]. Skeletal muscle atrophy has proven to be a significant problem in the area of physical therapy rehabilitation[12–15)]. However, changes in the levels of phosphorylation of cofilin and LIM kinases in immobilization-induced atrophy are not fully understood. Therefore, in the present study, we sought to demonstrate the changes in the phosphorylation of cofilin and LIM kinases in the gastrocnemius muscles of rats subjected to cast immobilization.

MATERIALS AND METHODS

Male Sprague-Dawley rats (n=15) were anaesthetized during the attachment of the plaster of paris casting material[12)]. Experimental procedures were performed as described

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in previous reports[12, 14]. Two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry were performed as reported in our previous studies[14, 15]. Furthermore, to measure the phosphorylation of cofilin and LIM kinases, gastrocnemius muscle strips were isolated after specific intervals of cast immobilization and snap-frozen in liquid nitrogen. The samples were then homogenized in a sample buffer. The homogenate was centrifuged and the supernatant was collected. Proteins (45–50 μg/lane) were separated on 12% polyacrylamide sodium dodecylsulfate (SDS) gels and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore; Bedford, MA, USA)[13]. Anti-cofilin and anti-LIM kinase 1 and 2 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibody-specific bands were quantified using an image analyzer (BioRad). The present investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol of this study was approved by the Committee of Ethics in Research of the University of Yongin, in accordance with the terms of Resolution 5-1-20, December 2006. Data are expressed as means±SEM. The data were statistically evaluated using Student's t test for comparisons between two time points and by ANOVA for multiple comparisons. A p value of < 0.05 was considered to be statistically significant.

RESULTS

The density of the cofilin expressed in the gastrocnemius muscles is shown in Fig. 1A. The phosphorylation of cofilin and LIM kinases was significantly diminished after 3, 7, 14, and 21 days of cast immobilization compared with the control group (n=3–4, Fig. 1B, Table 1). However, the expression of cofilin was significantly increased after 3, 14, and 21 days of cast immobilization compared with the control group (n=3–4, Fig. 1B, Table 1).

DISCUSSION

Our previous study demonstrated that the transcriptional regulation of the protein ligase, muscle RING finger-1 (MuRF-1), is upregulated in rat gastrocnemius muscles, and is involved in the development of cast immobilization-induced muscle atrophy[12]. Mitogen-activated protein kinases, such as extracellular signal-regulated kinase 1/2, stress-activated protein kinase/*c-Jun* NH_2-terminal kinase, and p38 mitogen-activated protein kinase, are simultaneously involved in muscle atrophy induced by immobilization and cell starvation[12, 13]. Furthermore, in another study, we demonstrated that cast immobilization increases the expression of myoglobin in rat gastrocnemius muscles[15]. These previous results suggest that changes in mitogen-activated protein kinase expression, causes myoglobin to adapt in response to physical stress, such as immobilization[12–15]. Meanwhile, cofilin, one of the actin-depolymerizing factor/cofilin family proteins, which includes cofilin-1, cofilin-2, and actin-depolymerizing factor (also called destrin) in mammals, binds to actin and plays a role in actin dynamics and reorganization, and other cellular functions[3, 16]. Cofilin activity is regulated by the phosphorylation of Ser-3 on its NH_2-terminal[17]. Phosphorylation of cofilin is also performed by LIM kinases (LIM kinase 1 and LIM kinase 2 in mammals), and inhibits actin binding and severing, and the depolymerizing activities of cofilin[9, 16]. LIM kinases are named after LIM motif-containing protein kinases, and the name is derived from an acronym of three transcription factors, Lin11, Isl-1, and Mec-3[18]. The kinases responsible for this phosphorylation are Rho-associated protein kinase

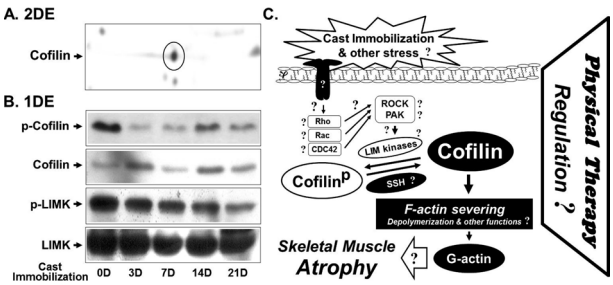

Fig. 1. Changes in phosphorylation of proteins, and a schematic representation of cellular responses to the immobilization. Proteomic (A) and immuno-blotting (B) analysis in the cast-immobilized skeletal muscle. 2DE and 1DE, two- and one-dimension gel electrophoresis; p-LIMK, phosphorylated LIM kinases; D, days; R, receptor; G-actin, globular actin; Rho-Rac-Cdc42, Rho family small GTPases; ROCK, Rho-associated protein kinase; PAK, p21-activated protein kinase; SSH, cofilin-specific phosphatase, slingshot.

Table 1. Changes in expression and phosphorylation of proteins in rat gastrocnemius muscles subjected to cast immobilization

Experimental period	p-Cofilin (%)	Cofilin (%)	p-LIM kinases (%)	LIM kinases (%)
0 day (control)	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
3 days	7.7±1.8*	246.7±14.5*	73.3±12.0*	99.7±0.3
7 days	19.0±4.9*	116.7±21.9	58.3±7.3*	98.7±4.2
14 days	33.3±10.1*	203.3±14.5*	60.0±7.6*	100.7±3.0
21 days	26.7±10.1*	173.3±12.0*	45.0±10.4*	98.0±4.4

Means±SEM. p, phosphorylated protein; LIM kinase, a serine kinase. The basal levels of proteins and phosphorylated proteins in controls (0 days) were considered to be 100%. *: vs. 0 day control, $p<0.05$

(ROCK) and p21-activated protein kinase (PAK), which are downstream kinases of the Rho family small GTPases such as Rho, Rac, and Cdc42[19–21]. Whereas, the dephosphorylation of cofilin is mediated by the cofilin-specific phosphatase, slingshot (SSH)[7, 8](Fig. 1C). Especially, cofilin is expressed in vascular smooth muscle cells and tissues, where it has been implicated in the regulation of cellular responses to reactive oxygen species (ROS), such as H_2O_2[3], and the progression of bladder cancer[22]. Although cofilin is identified in skeletal muscle using proteomic analysis[14], it has not previously been reported that phosphorylation of cofilin is related to muscle atrophy induced by cast-immobilization. In the present study, we have demonstrated for the first time that decrease of phosphorylation of cofilin and LIM kinases is associated with skeletal muscle atrophy induced by cast immobilization. However, further systematic studies covering electrotherapy, neurotherapy, hydrotherapy and others are needed to confirm the mechanisms of cofilin and LIM kinases in various muscle atrophy conditions[23–27] (Fig. 1C). In summary, the phosphorylation of cofilin and LIM kinases decreased in cast-immobilized rat gastrocnemius muscles. The present results suggest that cast immobilization-induced atrophy may be mediated by LIM kinase and cofilin in rat gastrocnemius muscles.

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