

Cofilin Phosphorylation Decreased by Serum-free Starvation with Low Glucose in the L6 Myoblasts

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Abstract. [Purpose] Many studies have been using cell culture models of muscle cells with exogenous cytokines or glucocorticoids to mimic atrophy in in vivo and in vitro tests. However, the changes in the phosphorylation of atrophy-related cofilin are still poorly understood in starved skeletal muscle cells. In this study, we first examined whether or not phosphorylation of cofilin is altered in L6 myoblasts after 3, 6, 12, 24, 48, and 72 hours of serum-free starvation with low glucose. [Methods] We used Western blotting to exam protein expression and phosphorylation in atrophied L6 myoblasts. [Results] L6 cell sizes and numbers were diminished as a result of serum-free starvation in a time-dependent manner. Serum-free starvation for 3, 6, 12, 24, 48, and 72 hours significantly decreased the phosphorylation of cofilin, respectively. [Conclusion] These results suggest that starvation-induced atrophy may be in part related to changes in the phosphorylation of cofilin in L6 myoblasts.

Key words: Cofilin, Serum-free starvation, L6 myoblasts

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INTRODUCTION

Starvation and other altered metabolic conditions such as immobilization, denervation, aging, and unloading states induces loss of muscle mass¹⁻⁴⁾. To study the signal transduction of atrophy in particular, various cell culture models have been developed⁵⁻⁷⁾. In many studies, exogenous cytokines such as TNF- α , glucocorticoids such as dexamethasone, and serum-free starvation of cultured cells have been used as atrophy models to confirm the mechanisms of whole skeletal muscle atrophy in vivo⁸⁻¹⁰⁾. The elevated degradation of proteins in skeletal muscle atrophy and serum-free starvation is commonly coupled with acti-

vation of the protein ligases such as muscle specific RING finger-1 (MuRF-1) and atrogin-1^{1, 4, 5, 11)}. Meanwhile, cofilin is a ubiquitously expressed protein in mammalian cells and thereby regulates the actin filament dynamics and reorganization and other functions¹²⁻¹⁴⁾. Furthermore, cofilin binds to actin molecules, changing fibrous actin to globular actin¹³⁾. This process is enabled by the dephosphorylation of cofilin by phosphatases^{12, 15)}. On the other hand, phosphorylation of cofilin abolishes the cofilin activity and inhibits its severing function^{12, 16)} (Fig. 1C). However, the changes in phosphorylation of cofilin in starvation-induced atrophy are not fully understood. Therefore, we investigated the changes in the phosphorylation of cofilin in L6 myoblasts during serum-free starvation with low glucose.

MATERIALS AND METHODS

L6 myoblasts from rat neonate skeletal muscle were separated into control and serum-free starvation groups¹⁾. The control group of L6 myoblasts was purchased from the American Type Culture Collection (Rockville, MD,

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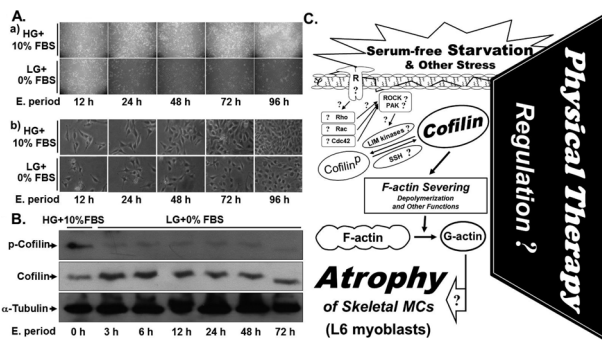


Fig. 1. Change in phosphorylation of protein and schematic representation of the cellular response caused by serum-free starvation with low glucose.

Morphologic (A-a \times 30, A-b \times 100) and immunoblotting (B) analyses in the starved L6 myoblasts. FBS, fetal bovine serum; h, hours; E. period, experimental period; HG, high-concentration glucose; LG, low-concentration glucose; p-Cofilin, phosphorylated cofilin; R, receptor; F-actin, fibrous actin; G-actin, globular actin; Rho-Rac-Cdc42, Rho family small GTPases; ROCK, Rho-associated protein kinase; PAK, p21-activated protein kinase; SSH, cofilin-specific phosphatases slingshot; Skeletal MCs, skeletal muscle cells.

USA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 200 mM glutamine, and 4,500 mg/L high-concentration D-glucose. The serum-free starvation group of L6 myoblasts grown to 60–70% confluence and undernourished in DMEM containing 1,000 mg/L low-concentration D-glucose without FBS for 3, 6, 12, 24, 48, and 72 h, respectively¹. After each experimental treatment, cells were lysed with an extraction buffer (20 mM HEPES, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na₃VO₄, 2.5 mM 4-nitrophenylphosphate, 0.5 mM PMSF, and one tablet of Complete Proteinase Inhibitor Cocktail [Roche, Indianapolis, IN, USA]). The morphological changes in L6 myoblasts with or without each experimental treatment were visualized with an inverted microscope (AE30/31, Motic Incorporation, Richmond, BC, Canada). To measure the phosphorylation of cofilin, the samples were then homogenized in a sample buffer. The homogenate was centrifuged, and the supernatant was collected. Proteins (30–45 μ g/lane) were separated on 12% polyacrylamide sodium dodecyl sulfate gels and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore; Bedford, MA, USA)². Anti-cofilin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody-specific bands were quantified using an image analyzer (Bio-Rad). The protocol for the 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 were expressed as means \pm SEM. The data were statistically evaluated using Student's t-tests for comparisons between pairs of groups and by ANOVA for multiple comparisons. A p value of < 0.05 was considered to be statistically significant.

Table 1. Changes in expression and phosphorylation of cofilin of L6 myoblasts during serum-free starvation with low glucose

Experimental period	Cofilin (%)	p-Cofilin (%)
0 hour (control)	100.0 \pm 0.0	100.0 \pm 0.0
3 hours	252.3 \pm 29.5*	20.7 \pm 6.1*
6 hours	242.7 \pm 20.7*	25.3 \pm 4.6*
12 hours	201.0 \pm 26.2*	24.7 \pm 6.3*
24 hours	198.7 \pm 22.9*	26.3 \pm 5.9*
48 hours	196.0 \pm 22.1*	24.3 \pm 5.4*
72 hours	176.3 \pm 14.4*	18.0 \pm 5.5*

Data were presented as the mean \pm SEM. p, phosphorylated protein. The basal levels of abundance and phosphorylation in controls (0 hour) were considered to be 100%. *Compared with the 0 hour control, p<0.05.

RESULTS

L6 cell sizes and numbers were diminished as a result of serum-free starvation in a time-dependent manner (Fig. 1A). Phosphorylation of cofilin was significantly decreased after 3, 6, 12, 24, 48, and 72 hours of starvation compared with those of the control groups (n=3–4, Fig. 1B, Table 1). However, the expression of cofilin was significantly increased after 3, 6, 12, 24, 48, and 72 hours of starvation compared with the expression of cofilin in the control groups (n=3–4, Fig. 1B, Table 1).

DISCUSSION

Skeletal muscle atrophy and joint contracture have proven to be significant orthopaedic problems in the area of physical therapy^{3, 17, 18}. The skeletal muscle is the largest organ with high plasticity in the human, comprising about 50% of the total body weight. Maintenance of muscle mass and neuromuscular function is important for activities daily living (ADL) in patients^{1, 2, 19}, and maintenance of muscle mass in particular is related in part to optimal nutrient absorption and use. In contrast, nutrient starvation and disuse have in part the potential to negatively impact muscle mass such as through skeletal muscle atrophy^{20–23}. Our previous study reported that the ubiquitin protein ligases MuRF-1¹ and atrogin-1 (data not shown), markers of muscle atrophy, are increased in atrophied gastrocnemius muscle strips and involved in the development of serum-free starvation-induced atrophy in L6 myoblasts. Simultaneously, extracellular signal-regulated kinase 1/2 (ERK1/2), stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK), and p38 mitogen-activated protein kinase (p38MAPK) are involved in atrophy caused by cast immobilization of a hind limb and serum-free starvation of L6 myoblasts^{1, 2}. Also, our previous report demonstrated that cast immobilization of rat gastrocnemius muscles increases the expression of tissue myoglobin²⁴. Meanwhile, cofilin in eukaryotic cells binds to actin and plays a role in actin dynamics and reorganization involved in cast immobilization-induced atrophy^{14, 25}. Phosphorylation of cofilin is achieved by LIM

kinases and thereby inhibits the actin binding, severing, and depolymerizing activities of cofilin^{16, 25)} (Fig. 1C). Furthermore, the kinases responsible for this phosphorylation are Rho-associated protein kinase and p21-activated protein kinase, which are downstream kinases of the Rho family small GTPases^{26–28)}. On the other hand, dephosphorylation of cofilin is mediated by the cofilin-specific phosphatases slingshot^{12, 15)} (Fig. 1C). Although cofilin is essential for maintenance of skeletal muscle mass¹²⁾, it has not been reported that phosphorylation of cofilin is related to atrophy caused by serum-free starvation in the area of physical therapy. However, further systematic studies in the area of physical therapy such as electrotherapy, neurotherapy, hydrotherapy, and others are needed to confirm the mechanism of cofilin under atrophic conditions^{29–31)} (Fig. 1C). In summary, the phosphorylation of cofilin was decreased in starved skeletal muscle cells. The present results suggest that serum-free starvation-induced atrophy may be in part mediated by cofilin from L6 myoblasts.

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