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Original Article

Decrease of PKB/Akt Phosphorylation is Partially Mediated by SAPK/JNK Activation in Serum-free L6 Myoblasts Starved with Low Glucose

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Abstract. [Purpose] Studies have been using cell cultures of muscle cells to mimic atrophy in *in vivo* and *in vitro* tests. However, changes in the activation of atrophy-related PKB/Akt is not fully understood in serum-free starved skeletal muscle cells. The purpose of the present study was to determine the change of PKB/Akt phosphorylation in L6 myoblasts under serum-free starvation conditions. [Methods] We used western blotting to examine PKB/Akt expression and phosphorylation in atrophied L6 myoblasts. [Results] The phosphorylation of PKB/Akt was significantly lower in L6 myoblasts under serum-free starvation than that of the control group. Serum-free starvation for 6, 12, 24, 36, 48, 72, 96, and 120 hours significantly decreased the phosphorylation of PKB/Akt. Furthermore, the decrease of PKB/Akt phosphorylation under serum-free starvation was partially restored by SP600125, an inhibitor of SAPK/JNK. [Conclusion] These results suggest that decrease of PKB/Akt phosphorylation due to serum-free starvation with low glucose is partially related to the activity of SAPK/JNK in L6 myoblasts.
Key words: PKB/Akt, Serum-free starvation, L6 myoblasts

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INTRODUCTION

Akt, also called protein kinase B (PKB/Akt), is a pleckstrin homology domain-containing serine/threonine kinase which is activated by various stimuli, such as growth factors and agonists[1–4)]. Akt has also been widely reported to have key roles in muscle hypertrophy and the prevention of atrophy[1, 3, 5)]. Our previous study demonstrated that the phosphorylation of PKB/Akt in atrophied muscle tissues significantly diminishes in a time-dependent manner in cast-immobilized rats[1)]. Starvation in culture induces loss of muscle mass[6–8)]. To study the signal transduction of atrophy, various cell culture models have been developed[9–12)]. The elevated degradation of proteins in skeletal muscle atrophy and serum-free starvation is coupled with the activation of atrophy markers such as muscle-specific RING finger-1 (MuRF-1) and the muscle atrophy F-box protein (MAFbx, also called atrogin-1), and these are greatly upregulated in the initiation and development of skeletal muscle atrophy[6–8)]. However, changes in the phosphorylation of PKB/Akt in starvation-induced atrophy and its temporal characteristics are not fully understood. Therefore, we investigated the changes in the phosphorylation of PKB/Akt in L6 myoblasts grown under serum-free starvation conditions with low glucose.

MATERIALS AND METHODS

The L6 myoblasts were purchased from the American

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Table 1. Changes in the expression and phosphorylation of PKB/Akt in L6 myoblasts grown under serum-free starvation with low glucose

Experimental period	PKB/Akt (%)	p-PKB/Akt (%)	Variable	PKB/Akt (%)	p-PKB/Akt (%)
0 hour	100.0±0.0	100.0±0.0	HG+10% FBS	100.0±0.0	100.0±0.0
6 hours	100.7±4.1	18.7±6.6*	LG+2% FBS	99.3±1.8	89.3±8.4
12 hours	99.3±1.2	11.3±4.5*	LG+0% FBS	98.7±0.9	7.7±2.4*
24 hours	98.7±2.6	10.3±3.5*	HG+10% FBS	100.0±0.0	100.0±0.0
36 hours	101.0±4.9	10.7±3.8*	LG+0% FBS	103.3±5.8	5.7±2.9*
48 hours	99.3±3.5	13.3±4.3*	SB203580 10 μM	102.7±2.3	7.0±3.8*
72 hours	100.7±5.8	11.3±4.1*	SP600125 10 μM	102.3±2.4	69.7±7.9*
96 hours	101.0±3.5	10.3±6.0*	LY294002 10 μM	103.3±2.3	4.3±2.4*
120 hours	102.3±4.1	10.7±5.9*			

Data are presented as the mean ± SEM. HG, glucose of high concentration; LG, glucose of low concentration; p, phosphorylated protein; PKB/Akt, protein kinase B/Akt; SB203580, an inhibitor of p38MAPK; SP600125, an inhibitor of SAPK/JNK; LY294002, an inhibitor of phosphatidylinositol 3-kinase. The basal levels of abundance and phosphorylation in the control (0 hour) were considered to be 100%. *: vs. 0 hour control, $p<0.05$

Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM glutamine and 4,500 mg/L D-glucose (high concentration). To induce serum-free starvation, groups of cells were grown to 60–70% confluence with undernourishment in DMEM containing 1,000 mg/L D-glucose (low concentration) without FBS for 6, 12, 24, 36, 48, 72, 96, and 120 h, respectively[7]. Groups of starved L6 myoblasts were also treated with SB203580 (10 μM, an inhibitor of p38MAPK), SP600125 (10 μM, an inhibitor of SAPK/JNK), and LY294002 (10 μM, an inhibitor of PI3 K) for 6 hours. After each experimental treatment, cells were lysed with an extraction buffer. To measure the phosphorylation of PKB/Akt, the samples were then homogenized in a sample buffer. The homogenate was centrifuged and the supernatant was collected. Proteins, 30–45 μg/lane, were separated on 12% polyacrylamide sodium dodecylsulfate gels and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore; Bedford, MA, USA)[1]. Anti-PKB/Akt antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). SB203580, SP600125, and LY294002 were purchased from Tocris Bioscience (Bristol, UK). Antibody-specific bands were quantified using an image analyzer (BioRad). Data were expressed as the means±SEM. The statistical evaluation of data was performed using GraphPad prism (GraphPad Software, San Diego, CA, USA), and Student's t-test for group comparisons and ANOVA for multiple comparisons. A p value of < 0.05 was considered statistically significant. 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.

RESULTS

The phosphorylation of PKB/Akt was significantly lower in L6 myoblasts grown under starvation conditions with low glucose and 0% FBS than that in the control group cultured with high glucose and 10% FBS (n=4; Table 1, Fig. 1A). Serum-free starvation with low glucose for 6, 12, 24, 36, 48, 72, 96, and 120 hours significantly decreased the phosphorylation of PKB/Akt (n=5; Table 1, Fig. 1B). Furthermore, the decrease of PKB/Akt phosphorylation in serum-free starvation with low glucose was partially restored by 10 μM SP600125, an inhibitor of SAPK/JNK (n=4; Table 1, Fig. 1C). However, the starvation did not influence the abundance of PKB/Akt expression in any group (n=4–5; Table 1, Fig. 1A, 1B, and 1C).

DISCUSSION

Maintenance of muscle mass and of muscle function are important for healthy life, and is important in the rehabilitation of musculoskeletal disease in the field of physical therapy[1, 7, 13, 14]. The maintenance mechanisms of muscle mass include anabolic and catabolic signal transductions via PKB/Akt, a protein with a critical role[1, 5, 10, 11]. PKB/Akt intermediates the signaling pathways that regulate cellular processes controlling growth, proliferation, and differentiation[2, 3, 11, 12], and supplementation with growth factors and nutrients increases the activity of PKB/Akt[10–12]. Supplementation encompasses intake of food, quality of diet, and the ability to optimally use ingested nutrients in the maintenance of muscle mass[15, 16]. In contrast, as muscle mass decreases, there is an accompanying loss in muscle strength and use of the nutrients that contributes to reduced muscle function and quality of activities of daily living (ADL). Malnutrition and chronic diseases such as diabetes mellitus, heart failure, and chronic obstructive pulmonary disease are also directly associated with a dramatic reduction in the phosphorylation of PKB/Akt with decrease of muscle mass[15, 17, 18]. Our previous study reported that increases in atrophy markers and a decrease in PKB/Akt phosphorylation occur in gastrocnemius muscle strips atrophied by cast-immobilization, and PKB/Akt phosphorylation is involved in the development of serum-free starvation-induced MuRF-1 expression in L6 myoblasts[1, 7]. Extracellular sig-

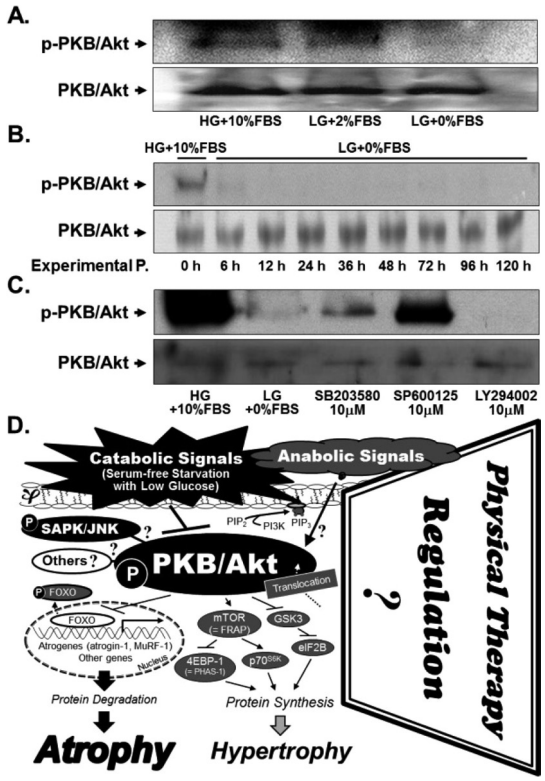

Fig. 1. Change of phosphorylation of PKB/Akt and a schematic representation of the cellular response induced by serum-free starvation with low glucose in L6 myoblasts
PKB/Akt, protein kinase B/Akt; FBS, fetal bovine serum; h, hours; Experimental P, experimental period; HG, glucose of high concentration; LG, glucose of low concentration; SAPK/JNK, stress-activated protein kinase/*c-Jun* NH_2-terminal kinase; SB203580, an inhibitor of p38MAPK; SP600125, an inhibitor of SAPK/JNK; LY294002, an inhibitor of phosphatidylinositol 3-kinase; MuRF-1, muscle specific RING finger-1; FOXO, Forkhead box O transcription factors; mTOR, mammalian target of rapamycin; FRAP, FKBP12-rapamycin-associated protein; GSK3, glycogen-synthase kinase 3; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2]; PIP_3, phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P_3]; p-PKB/Akt, phosphorylated PKB/Akt; 4EBP-1, 4E-binding protein 1; PHAS-1, phosphorylated heat- and acid-stable protein 1; eIF2B, eukaryotic initiation factor 2B; $p70^{S6K}$, p70 S6 kinase

nal-regulated kinase 1/2 (ERK1/2), stress-activated protein kinase/*c-Jun* NH_2-terminal kinase (SAPK/JNK), and p38 mitogen-activated protein kinase (p38MAPK) are concurrently involved in atrophy induced by cast-immobilization and the starvation of skeletal muscle cells[1, 7]. Furthermore, our previous report demonstrated that cast-immobilization of rat gastrocnemius muscles increases the expression of muscle myoglobin[19]. It has recently been reported that cofilin in eukaryotic cells binds to actin and plays a role in actin dynamics and reorganization in cast immobilization- and starvation-induced atrophy in rat gastrocnemius muscles and L6 myoblasts[20–22]. However, further systematic and scientific studies in the fields of electrotherapy, neurotherapy, hydrotherapy and others are needed to confirm the mechanisms of PKB/Akt in atrophied muscle strips and cells[23–27] (Fig. 1D). In summary, the phosphorylation of PKB/Akt decreased in starved L6 skeletal muscle cells. The present results suggest that serum-free starvation-induced atrophy is partially mediated by PKB/Akt via the SAPK/JNK pathway in L6 myoblasts.

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