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DJ-1 protects against undernutrition-induced atrophy through inhibition of the MAPK–ubiquitin ligase pathway in myoblasts

Junghwan Kim [a,1], Kyung-Jong Won [b,1], Seung Hyo Jung [b], Kang-Pa Lee [b], Sun Bo Shim [c], Mee-Young Kim [a], Ju-Hyun Kim [d], Jeong-Uk Lee [e], Bokyung Kim [b,*]

[a] Department of Physical Therapy, College of Public Health & Welfare, Yongin University, Yongin-city 449-714, South Korea
[b] Department of Medicine, Konkuk University School of Medicine, Chungju-city 380-701, South Korea
[c] Laboratory Animal Resources Division, National Institute of Food and Drug Safety Evaluation, Korea Food & Drug Administration, Cheongwon-gun 363-700, South Korea
[d] Department of Physical Therapy, College of Health Welfare, Wonkwang Health Science University, Iksan 570-750, South Korea
[e] Department of Physical Therapy, College of Health Science, Honam University, Gwangju 506-714, South Korea

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ABSTRACT

Aims: The purpose of this study is to explore whether antioxidant DJ-1 protein affects the atrophy of skeletal muscle cell induced by undernutrition.
Main methods: To determine cell atrophic responses, L6 cell line and skeletal primary cells from mouse hind limbs were cultivated under condition of FBS-free and low glucose. Changes of protein expression were analyzed using Western blot. Overexpression and knockdown of DJ-1 was performed in cells to assess its influence on cell atrophic responses.
Key findings: Undernutrition decreased cell size and increased the abundance of oxidized form and total form of DJ-1 protein in L6 myoblasts. The undernourished cells revealed an elevation in the expression of muscle-specific RING finger-1 (MuRF-1) and atrogin-1, and in the phosphorylations of p38 mitogen-activated protein kinase (MAPK) and stress-activated protein kinase/c-*Jun* N-terminal kinase compared with control groups. Moreover, DJ-1-knockout mice showed a decrease in cell size and an enhancement in the expression of MuRF-1 and atrogin-1, as well as in the phosphorylation of MAPKs in gastrocnemius muscles; these changes were also observed in L6 cells transfected with siRNA of DJ-1. On the other hand, L6 cells overexpressing full-length DJ-1 did not exhibit the alterations in cell size and ubiquitin ligases seen after undernourished states of control cells. Myotubes differentiated from L6 cells also showed elevated expression of MuRF-1 and atrogin-1 in response to undernutrition.
Significance: These results suggest that DJ-1 protein may contribute to undernutrition-induced atrophy via MAPKs/ubiquitin ligase pathway in skeletal muscle cells.

1. Introduction

Muscle atrophy is caused through various condition including immobilization, denervation, aging and bed rest, which can contribute, both directly and indirectly, to a more generalized loss of muscle function [1–3]. Muscle atrophy has widely been reported to decrease muscle mass via decrease of contractile proteins [4], capillary densities [5,6], enzymatic activities [7], and mechanical parameters [8]. The decrease of capillary density by muscle atrophy is directly correlated with undernutrition or malnutrition in skeletal muscle [6,9,10]. Furthermore, the elevated degradation of intracellular proteins in atrophied skeletal muscle is commonly coupled with activation of the ubiquitin-dependent protease pathway, and ubiquitin ligases such as muscle-specific RING finger-1 (MuRF-1) and atrogin-1 (also called muscle atrophy F-box) are upregulated in both the initiation and the progression of atrophy [11,12]. In addition, changes in the abundance of phosphorylated forms of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1/2, stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) and p38 MAPK, have been implicated in skeletal muscle atrophy [13,14].

DJ-1, a multifunctional protein responding to oxidative stress, has known to be able to participate in the regulation of cell function in a variety of cells [15]. Changes in DJ-1 level have been implicated in the pathogenesis of various diseases, including Alzheimer's disease and Pick's disease [16]. Loss of DJ-1 is associated with early-onset autosomal

Abbreviations: DJ-1$^{-/-}$, DJ-1-knockout; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MuRF-1, muscle-specific RING finger-1; NC, NCBI; Ov-DJ-1, DJ-1-overexpressed cells; Ox-DJ-1, oxidized DJ-1; PD, Parkinson disease; SAPK/JNK, stress-activated protein kinase/c-*Jun* N-terminal kinase; siRNA, small interfering RNA; Syk, spleen tyrosine kinase.

* Corresponding author at: Department of Physiology, School of Medicine, Konkuk University, 322 Danwol-dong, Chungju 380-701, South Korea.
E-mail address: bkkim2@kku.ac.kr (B. Kim).

[1] J.K. and K.J.W. contributed equally to this work.

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recessive Parkinson disease (PD) [17]. Further, PD leads to severe locomotor dysfunction and decreases in isometric pinch grip force, suggesting that a low activity of DJ-1 may result in widespread skeletal muscle dysfunction [18,19]. Moreover, DJ-1 can bind to multiple ligands involved in transcriptional regulation, protease or redox-dependent chaperone activity, RNA–protein interactions, apoptosis and SUMOylation, all of which have been shown to play a role in skeletal muscle activity [20,21]. DJ-1 therefore appears to be an important player in skeletal muscle function in both health and disease, although its mechanistic roles are yet to be determined.

Reactive oxygen species (ROS) are a class of signaling molecules that play important roles in various cellular functions and diseases [22]. In skeletal muscle, it has been shown that ROS can be induced through activation of the NADPH oxidase system following prolonged muscle disuse [23,24] and also that ROS-activated signaling can contribute to skeletal muscle atrophy itself [25,26]. In addition, antioxidants prevented the skeletal muscle disorders related to atrophy [27]. DJ-1 acts as a redox sensor and ROS scavenger in vivo, which in turn can lead to various oxidation states of the protein [28,29]. It was also suggested that DJ-1 may prevent ROS accumulation by regulating the level of cellular glutathione and other antioxidants [30]. Taken together, these results suggest that DJ-1 may play a central role in skeletal muscle atrophy. To thus demonstrate a possible involvement of DJ-1 protein in development of skeletal muscle atrophy, especially undernutrition-induced muscle atrophy, we examined correlation between DJ-1 and atrophy in response to undernutrition in L6 cells and in skeletal muscle primary cells from DJ-1-knockout (DJ-$1^{-/-}$) mice.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), horse serum, penicillin, and streptomycin were obtained from Hyclone (Logan, UT, USA). Dulbecco's modified eagle's medium (DMEM) were purchased from GIBCO (Carlsbad, CA, USA). Total and phosphorylated p38 MAPK and SAPK/JNK antibodies were purchased from Cell Signaling (Beverly, MA, USA). MuRF-1, atrogin-1 and total DJ-1 antibodies were from Santa Cruz (Santa Cruz, CA, USA). An antibody to the oxidized form of DJ-1 was obtained from AbD Serotec (Raleigh, NC, USA). The β-actin antibody and other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Isolation of skeletal muscle primary cell from DJ-1 knockout animals

Our 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 2011) and all experiments and animal care conformed to the institutional guidelines established by Konkuk University, Korea. Male and female DJ-1 homozygous knockout (B6.Cg-Park7tm1shn/J, 25–30 g; n = 8) and wild-type (DJ-$1^{+/+}$; n = 8) mice with the same background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). To confirm DJ-1 gene depletion in mice, the distal tips of tails were obtained from DJ-$1^{-/-}$ and DJ-$1^{+/+}$. A genotyping using polymerase chain reaction (PCR) for DJ-$1^{-/-}$ confirmation was performed with the following primers: DJ-1 forward, 5′-GCT GAA ACT GTG CCA TGT GA-3′; DJ-1 reverse, 5′-TGC TAA AGC GCA TGC TCC AGA CT-3′; Mutant Neo, 5′-TGG ATG TGG AAT GTG TGC GAG-3′. The expression of DJ-1/park7 protein was confirmed in aortic strips using Western blotting analysis.

The animals were scarified by CO_2 inhalation and skeletal muscle primary cells were isolated from their hind limbs. Briefly, the gastrocnemius skeletal muscles were removed rapidly and carefully from the limbs of animals and placed in cold Krebs solution (containing, in mM: NaCl 118.0; KCl 4.8; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_3$ 24.9; Glucose 10.0; KH_2PO_4 1.2). After washed with fresh Krebs solution, the skeletal muscles were minced into small pieces (about 1 to 2 mm^3) in DMEM supplemented with 20% FBS, 10% horse serum and 100 U/ml penicillin, and 100 μg/ml streptomycin. The 10 pieces of minced muscle tissues were plated on culture dish with the culture medium and cultivated for 48 h at 37 °C under a humidified 95% air/5% CO_2 mixture (vol/vol). After elimination of the supernatants and muscle tissue from the culture dishes, the adhering bead-like round cells, which was observed to about 30% confluence, were re-cultivated in fresh culture medium for 24 h at 37 °C under a humidified 95% air/5% CO_2 mixture (vol/vol), and thereafter culture medium was changed every 24 h to purify the cells by detection of morphology until the cells reached about 60–70% confluence.

2.3. Cell cultures

The rat skeletal muscle L6 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). The L6 cells and skeletal muscle primary cells purified and isolated were separated into groups of control and undernutrition. The control group was cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM glutamine and 4500 mg/L D-glucose. While undernutrition group was grown to 60–70% confluence and undernourished in DMEM containing 1000 mg/L D-glucose without FBS for 24 h. After each experimental treatment, cells were lysed with an extraction buffer containing 20 mM HEPES, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 2.5 mM 4-nitrophenylphosphate, 0.5 mM PMSF, and one tablet of complete proteinase inhibitor cocktail (Roche, Indianapolis, IN, USA).

2.4. Measurement of the cell size

The morphological changes in L6 cells were visualized with an inverted microscope (AE30/31, Motic Incorporation, Canada) and cell size was measured with Infinity 1 CMOS cameras and Infinity v5.0.2 analyze software (Lumenera Incorporation, Canada). For immunohistochemical analysis, the specimens were blocked with phosphate buffered saline (PBS) containing 5% serum for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C, and then treated with fluorescent-labeled secondary antibodies (Vectastain Universal Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Images were captured with a confocal microscope system (FV-1000 Spectral, Olympus, Japan) and quantified using Fluoview software (FV10-ASW, Olympus).

2.5. Myotubes

To induce differentiation into myotubes, L6 cells (4×10^6/ml) were plated in culture dish and incubated overnight in culture medium, containing 2% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM glutamine and 4500 mg/L D-glucose, at 37 °C under a humidified 95% air/5% CO_2 mixture (vol/vol). Cells were replaced with differentiation medium and were incubated for 5 days, during which time myotube formation occurred. The differentiation medium was changed every 24 h. The differentiated cells from passages 3–4 were used in the test. To identify myotubes, the cells were fixed and immuno-stained with anti-myosin heavy chain-2 protein antibody.

2.6. Overexpression and knockdown of DJ-1 in cells

An expression vector containing the whole coding sequence of DJ-1 tagged with V5 epitope was constructed using pcDNA3.1/nV5-DEST™ Gateway™ Vector Pack (Invitrogen, California CA, USA). Briefly, NP_476484 (GenBank; NM-057143) was amplified by PCR using the mRNA sequence from GenBank. L6 cells were electroporated using the Basic Nucleofector Kit (AMAXA, Cologne, Germany) with the AMAXA Nucleofector II according to the manufacturer's instructions. Transfected cells were cultured for 16 h. The expression of transfected DJ-1 was evaluated by immunoblotting analysis.