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ScienceDirect
Biochemical and Biophysical Research Communications 357 (2007) 314–318
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Hypertrophy and atrophy inversely regulate Caveolin-3 expression in myoblasts

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Received 23 March 2007
Available online 2 April 2007

Abstract

Caveolin-3 (Cav-3) is a muscle-specific membrane protein crucial for myoblast differentiation, as loss of the protein due to mutations within the gene causes an autosomal dominant form of limb girdle muscular dystrophy 1-c. Here we show that along with p38 activity the PI3-kinase/AKT/mTOR pathway is required for proper Cav-3 up-regulation during muscle differentiation and hypertrophy, as confirmed by the marked increase of Cav-3 expression in hypertrophied C2C12 cells transfected with an activated form of AKT. Accordingly, Cav-3 expression was further increased during hypertrophy of L6C5 myoblasts treated with Arg^8-vasopressin and in hypertrophic muscles of MLC/mIGF-1 transgenic mice. In contrast, Cav-3 expression was down-regulated in C2C12 myotubes exposed to atrophic stimuli such as starvation or treatment with dexamethasone. This study clearly suggests that Cav-3 expression is causally linked to the maturation of muscle phenotype and it is tightly regulated by hypertrophic and atrophic stimuli.

Keywords: Caveolin 3; Hypertrophy; AKT; Igf-1; Vasopressin; Atrophy; Starvation; Dexamethasone

Mammalian caveolins consist of three integral membrane proteins, termed Cav-1, Cav-2, and Cav-3 [1–4], that represent the main structural elements of caveolae, 50–100 nm invaginations of the plasma membrane [5–7]. Caveolins, acting as scaffolding proteins, are able to concentrate lipids (cholesterol and glycosphingolipids [8–10]), signalling proteins (G-proteins, H-Ras, nitric oxide synthase, Src-like kinases [8,11–16]), and structural proteins (dystroglycan, M-phosphofructokinase and dysferlin [17–20]) in the caveolae.

Caveolins 1 and 2 have a similar tissue distribution, and form hetero-oligomeric complex in different cell types, as adipocytes, endothelial cells, pneumocytes, and fibroblasts [21], whereas Cav-3 expression is restricted to skeletal muscle and heart [18]. During myotube formation Cav-3 expression is strongly up-regulated by activation of p38 pathway [22], resulting localized at the plasma membrane of muscle fibers (sarcolemma) in complex with dystrophin and its associated glycoproteins [18]. The crucial role of Cav-3 in muscle was clearly demonstrated by the finding that mutations in the Cav-3 gene are responsible for an autosomal dominant form of limb girdle muscular dystrophy 1-c [23]. These mutations cause the loss of Cav-3 protein, as the mutated Cav-3 proteins associated with the endogenous wild type protein undergo proteosomal degradation [24]. Interestingly, the overexpression of Cav-3 in muscle mice results in a Duchenne-like phenotype [25], clearly demonstrating that a tight regulation of Cav-3 expression is crucial for proper myofiber organization.

In this study we demonstrate that the activation of both p38 and PI3-kinase/Akt/mTOR pathways is required for proper Cav-3 up-regulation during muscle differentiation.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HS, horse serum; PBS, phosphate buffer solution; BSA, bovine serum albumin.

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.03.148

Subsequently, we analyzed the expression pattern of Cav-3 in both muscle hypertrophy and atrophy. Our result suggest that Cav-3 is inversely regulated during these processes, as in vitro and in vivo hypertrophy leads to increased Cav-3 expression, whereas myotube atrophy impairs Cav-3 expression.

Materials and methods

Cell culture and pharmacological treatments. The mouse C2C12 myoblasts were maintained at subconfluent density at 37 °C in 5% CO_2 and cultured in DMEM high glucose (Sigma–Aldrich) supplemented with 10% FBS (Sigma–Aldrich) and 100 μg/ml penicillin–streptomycin antibiotic (Sigma–Aldrich), defined as growth medium (GM). Confluent cells were shifted to differentiation medium (DM) containing DMEM supplemented with 2% HS and the medium was changed every two days. Pharmacological treatments of myoblasts were performed every day using 10 μM SB239063 (Sigma–Aldrich) to inhibit p38 activity, 10 μM LY294002 (Sigma–Aldrich) to inhibit PI3-kinase activity, 5 ng/ml rapamycin (Sigma–Aldrich) to inhibit mTOR activity. The rat L6C5 myoblasts were seeded at 25,000/cm^2 in GM, and twenty-four hours after plating the cultures were shifted to serum-free medium consisting of DMEM supplemented with 1% fatty acid-free BSA (Sigma–Aldrich) and treated with synthetic 0.1 μM Arg^8-vasopressin (AVP, Sigma–Aldrich) for different time-points.

To induce myotube atrophy, C2C12 cells grown in DM were either the serum, glucose, and aminoacid starved or added with 100 μM dexamethasone (Sigma–Aldrich) at the indicated different time-points.

Cell staining and myotube quantification. To visualize myotubular structures, cells were washed three times in PBS before fixing for 10 min in 100% methanol at −20 °C. Cells were stained with Giemsa reactive (Sigma–Aldrich) for 2–3 h and again washed in PBS. To quantify the myotube diameter, 10 fields were chosen randomly and 10 myotubes were measured per field. The average diameter per myotube was the mean of 10 measurements taken along the length of the myotube.

Stable transfections. To obtain C2C12 myoblasts expressing the constitutive active form of AKT, the cells were transfected using a pBABE vector in which a myristoylated AKT was cloned. After transfection by Lipofectamine 2000 reagent, the mix of stable transfectants were obtained after 10 days selection in puromycin antibiotic (2 μg/ml, Sigma–Aldrich) and used by few passages.

Western blot analysis. Protein concentration was obtained by bicinchoninic acid assay (Pierce). For Cav-3 immunoblotting, myoblast cells were washed twice with PBS and lysed 30 min at 4 °C in a buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, and 1% Triton X-100 added with a mix of protease inhibitors (Roche Molecular Biochemicals). Samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Pellet (insoluble fraction) was resolved by SDS–PAGE (12% acrylamide) and a mouse monoclonal antibody against Cav-3 was used (clone 26, BD Transduction Laboratories). Muscle tissues from wild type and transgenic MLC/mIgf-1 mice [26] were homogenized in presence of liquid nitrogen and sonicated in a buffer containing 10 mM Tris (pH 8.0), 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100 and 60 mM octyl-glucoside added with a mix of protease inhibitors. In addition, samples were centrifuged at 13,000*g* for 10 min at 4 °C to remove insoluble proteins. Soluble proteins were then resolved by SDS–PAGE (12% acrylamide) and transferred to nitrocellulose membranes before Cav-3 detection. For the detection of GATA-2 and the phosporylated forms of AKT and P70S6 K, myoblast cells were harvested at 4 °C in RIPA lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS in 50 mM NaCl, 20 mM Tris–HCl pH 7.6) containing a mix of protease inhibitors. Lysates were cleared by centrifugation at 12,000*g* for 15 min at 4 °C before resolving the surnatants by SDS–PAGE (10% acrylamide). GATA2 was detected using a mouse monoclonal antibody (clone CG2-96, Santa Cruz Biotechnology). The phosphorylated forms of AKT (Ser^{473}) and P70S6 K (Thr^{389}) were detected using rabbit polyclonal antibodies (Cell Signalling). An antibody against alpha tubulin (Sigma–Aldrich) was used to normalize the loading. Western blots were revealed with enhanced chemiluminescence (Chemicon).

Statistics. All of the data are expressed as means ± SE Statistical significance was determined using *t*-Student's analysis. A p value of <0.05 was considered significant.

Results and discussion

PI3-kinase/AKT/mTOR and p38 pathways are both required for Cav-3 expression during myoblast differentiation

Cav-3 has long been known to be required for proper myoblast differentiation, as its targeted down-regulation is sufficient to inhibit myotube formation in C2C12 cells [18,22]. It has been previously reported that activation of p38 pathway is required to up-regulate Cav-3 expression during myotube formation [22]. In this study, we further explored the effects of PI3-kinase/AKT/mTOR pathway inhibition on Cav-3 expression, being the activation of this pathway crucial for C2C12 myoblast differentiation [27,28]. As expected, the inhibition of either p38 activity or PI3-kinase/AKT/mTOR pathway by pharmacological treatments impaired myotube formation of C2C12 cells (data not shown). As shown in Fig. 1, terminally differentiated C2C12 cells, cultured for 4 days in differentiating medium (DM), exhibited up-regulation of Cav-3 expression. In contrast, the pharmacological inhibition of each p38, PI-3 kinase and mTOR activity by addition of either 10 μM SB239063, 10 μM LY294002 or 5 ng/ml rapamycin delayed and significantly decreased Cav-3 expression. These data suggest that the activation of p38 and PI3-kinase/AKT/mTOR pathways are both necessary to determine the physiological Cav-3 up-regulation during the stages of myotube formation.

In vitro and in vivo myotube hypertrophy enhances Cav-3 expression

Skeletal muscle hypertrophy plays an important role during post-natal development occurring in response to muscle overload and resulting in an increase of transla-

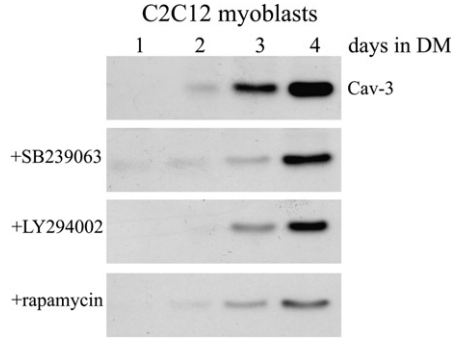

Fig. 1. Inhibition of both p38 and PI3-kinase/AKT/mTOR pathways down-regulates Cav-3 expression. C2C12 myoblasts were differentiated for 4 days in presence of the indicated treatments and subjected to Western blot analysis of Cav-3 expression using a monoclonal antibody.