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Research Article

Hypertrophic response of Duchenne and limb-girdle muscular dystrophies is associated with activation of Akt pathway

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Abbreviations:
Akt/PKB, protein kinase B
DG, dystroglycan
DGC, dystrophin–glycoprotein complex
DMD, Duchenne muscular dystrophy
GSK, glycogen synthase kinase 3
GS, glycogen synthase
LGMD, limb-girdle muscular dystrophy
mTOR, mammalian target of rapamycin
PI(3)K, phosphoinositide 3-OH kinase
SG, sarcoglycan

ABSTRACT

Dystrophic muscle undergoes repeated cycles of degeneration/regeneration, characterized by the presence of hypertrophic fibers. In order to elucidate the signaling pathways that govern these events, we investigated Akt activation in normal and dystrophic muscle. Akt is activated in neonatal muscle and in actively dividing myoblasts, supporting a developmental role for Akt signaling. Akt activation was detected at very early, prenecrotic stages of disease pathogenesis, and maximal activation was observed during peak stages of muscle hypertrophy. Duchenne muscular dystrophy patients exhibit a similar pattern of Akt activation. Mice with sarcoglycan-deficient muscular dystrophy possess more severe muscle pathology and display elevated Akt signaling. However, the highest levels of Akt activation were found in dystrophin–utrophin-deficient muscle with very advanced dystrophy. We propose that Akt may serve as an early biomarker of disease and that Akt activation mediates hypertrophy in muscular dystrophy. Current investigations are focused on introducing constitutively active and dominant-negative Akt into prenecrotic *mdx* mice to determine how early modification of Akt activity influences disease pathogenesis.

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Introduction

The most well-characterized forms of muscular dystrophy involve mutations in the genes encoding components of the dystrophin–glycoprotein complex (DGC) (for review, see Ref. [1]). Integral and peripheral membrane components of the DGC include dystrophin, the dystroglycans (α- and β-DG), the sarcoglycans (α-, β-, γ-, and δ-SG), sarcospan, and the syntrophins [2–5]. The DGC is localized to the sarcolemma and forms a physical linkage between the extracellular matrix and the intracellular actin cytoskeleton. Dystrophin binds to β-DG and tethers the DGC to the intracellular actin cytoskeleton [2,3]. The extracellular matrix protein laminin-2 binds to the peripheral membrane protein α-DG, thereby providing structural integrity to the sarcolemma during muscle contraction [3,6]. Primary mutations in the dystrophin gene are responsible for causing Duchenne muscular dystrophy (DMD) [7–9]. Genetic mutations in either α-, β-, γ-, and δ-SG lead to autosomal-recessive limb girdle muscular dystrophy (AR-LGMD) (for review, see [10]). Absence of either dystrophin or any one of the SG proteins causes DGC instability, leading to sarcolemma damage and cycles of fiber degeneration/regeneration.

It is well established that the DGC provides structural stability to the sarcolemma during contraction. However, several lines of evidence suggest that the DGC may also play a role in cell signaling. First, the DGC is associated with several signaling molecules, including Grb2 [11], nNOS [12,13], caveolin-3 [14,15], dystrobrevin [16,17] as well as regulatory kinases [18,19]. Second, gene expression studies reveal that several signaling pathways are activated in young (pre-phenotypic) dystrophin-deficient muscle [20]. Third, results from cultured muscle cells support the hypothesis that the SG subcomplex participates in bidirectional signaling with the integrins [18]. Lastly, treatment of cultured mouse myoblasts with antibodies that block dystroglycan–laminin interactions causes apoptosis accompanied by perturbations in Akt signaling [21]. The findings that Akt activation may be somehow linked to the DGC complex is intriguing given that recent investigations have demonstrated the importance of the phosphoinositide 3-kinase (PI(3)K/Akt) pathway in regulation of muscle hypertropy [22]. In DMD patients, the regenerative capacity of muscle fibers eventually diminishes and myofibers undergo atrophy and necrosis [23,24]. The dystrophin-deficient mouse serves as a model of DMD. These mice are phenotypically normal at birth but undergo muscular degeneration starting at 3 weeks of age. Muscles from *mdx* mice undergo rounds of degeneration and regeneration accompanied by hypertrophy. Eventually, *mdx* mice die prematurally at ~78 weeks of age, when regeneration can not surpass degeneration. It has been proposed that the robust regenerative capacity of *mdx* mice compared to DMD patients is responsible for their relatively mild phenotype [25–28]. Thus, understanding signaling mechanisms that mediate this regenerative, hypertrophic response in *mdx* tissue may help to identify key molecules that could serve as therapeutic targets.

Akt, also known as protein kinase B (PKB), is a serine–threonine kinase that is stimulated by a number of receptor tyrosine kinases at the cell surface [29]. Activation of the PI(3)K/Akt signaling pathway is a key modulator of skeletal muscle hypertrophy both in vitro [22,30] and in vivo [31–33]. Overexpression of insulin-like growth factor (IGF-1) induces Akt activation and rescues muscle atrophy in vivo [31]. In addition, transgenic IGF-1 overexpression in a dystrophin-deficient mouse model has been shown to increase muscle mass and force generation compared to non-transgenic dystrophin-deficient controls [32]. Taken together, this evidence suggests that the PI(3)K/Akt pathway plays an important role in IGF-1 and load-induced muscle hypertrophy. Modulation of this pathway may be of therapeutic value for DMD. In the present study, we investigate effects of dystrophin- and sarcoglycan deficiency on the PI(3)K/Akt pathway.

Materials and methods

Cell culture and growth curve

C2C12 myoblasts, strain C3 H (American Type Cell Culture, Manassas, VA), were maintained in Dulbecco's modified essential medium (DMEM, Mediatech, Herndon, VA). Media were supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin–streptomycin (Mediatech, Herndon, VA). Cells were trypsinized after reaching 90% confluency on a 150-mm plate. Cells were then washed in phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (1.4 mM NaCl, 0.27 mM KCl, 1 mM Na_2HP0_4, and 0.18 mM dibasic phosphate, pH 7.4). 1.5×10^5 cells were then plated onto 100-mm gelatin coated plates containing DMEM media. 2 h postplating, media was changed and this time was designated as time = zero. At the zero time point and every 6 h thereafter, three 100-mm plates were trypsinized, washed, and then counted using a hemocytometer. Cells were then frozen at –80°C until analysis. At the termination of the time course experiment, frozen cells were lysed in modified RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM *N*-ethylmaleimide, 50 mM sodium fluoride, 2 mM α-glycerophosphate, 1 mM sodium orthovanadate, 100 nM okadaic acid, 5 nM microcystin LR, and 20 mM Tris–HCl, pH 7.6) supplemented with protease inhibitors (0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). Following incubation for 1 h at 4°C, cell lysates were clarified by centrifugation at 15,000×*g* for 15 min.

Animal models

Wild-type (C57BL/6) and *mdx* breeder pairs were purchased from Jackson Laboratories (Bar Harbor, ME). Heterozygous δ-sarcoglycan (Sgcd) knockout males (Jackson Laboratories, Bar Harbor, ME) were bred with wild-type (C57BL/6) females until homozygous Sgcd mice [34] were obtained. All mice were housed in the UCLA Life Sciences Vivarium. Mice at various ages were euthanized by CO_2 followed by cervical dislocation. To examine *mdx* disease progression, three mice were analyzed at 2 weeks (prenecrotic stage), 4 weeks (peak necrotic stage), and 41–59 weeks (hypertrophic stage) of age. Homozygous δ-sarcoglycan knockout mice were analyzed at 4 weeks of age. Skeletal muscles were harvested and snap frozen in liquid nitrogen. 13-week wild-type (F1B) and δ-sarcoglycan-deficient (BIO14.6) hamster skeletal muscles were obtained from Bio