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Halofuginone promotes satellite cell activation and survival in muscular dystrophies

Hila Barzilai-Tutsch [a], Anna Bodanovsky [a], Hadar Maimon [a], Mark Pines [b], Orna Halevy [a,*]

[a] Department of Animal Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel
[b] Institute of Animal Science, The Volcani Center, Bet Dagan 52505, Israel

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ABSTRACT

Halofuginone is a leading agent in preventing fibrosis and inflammation in various muscular dystrophies. We hypothesized that in addition to these actions, halofuginone directly promotes the cell-cycle events of satellite cells in the *mdx* and *dysf*$^{-/-}$ mouse models of early-onset Duchenne muscular dystrophy and late-onset dysferlinopathy, respectively. In both models, addition of halofuginone to freshly prepared single gastrocnemius myofibers derived from 6-week-old mice increased BrdU incorporation at as early as 18 h of incubation, as well as phospho-histone H3 (PHH3) and MyoD protein expression in the attached satellite cells, while having no apparent effect on myofibers derived from wild-type mice. BrdU incorporation was abolished by an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated protein kinase, suggesting involvement of this pathway in mediating halofuginone's effects on cell-cycle events. In cultures of myofibers and myoblasts isolated from *dysf*$^{-/-}$ mice, halofuginone reduced Bax and induced Bcl2 expression levels and induced Akt phosphorylation in a time-dependent manner. Addition of an inhibitor of the phosphinositide-3-kinase/Akt pathway reversed the halofuginone-induced cell survival, suggesting this pathway's involvement in mediating halofuginone's effects on survival. Thus, in addition to its known role in inhibiting fibrosis and inflammation, halofuginone plays a direct role in satellite cell activity and survival in muscular dystrophies, regardless of the mutation. These actions are of the utmost importance for improving muscle pathology and function in muscular dystrophies.

1. Introduction

The adult muscle tissue is composed of terminally differentiated mature myofibers [reviewed in 1,2]. In cases of acute stress such as injury, or under myopathic conditions, the myofibers' ability to regenerate and repair relies solely on the myogenic capacity of the muscle progenitor cells, the satellite cells [reviewed in 3]. These cells, normally quiescent, are situated in a niche between the sarcolemma and the basal lamina of the myofiber. In response to stress (e.g., mechanical stress, injuries, myopathies), appropriate stimulatory signals such as hepatocyte growth factor and nitric oxide (NO) [4–6] activate the satellite cells from their quiescent state. The cells are driven into the cell cycle and after several cell divisions, they exit the cell cycle and undergo myogenic differentiation, subsequently fusing with pre-existing or new myofibers [reviewed in 2,7].

In Duchenne muscular dystrophy (DMD), characterized by near absence of the protein dystrophin in skeletal muscles [8], the myofibers undergo repetitive cycles of degeneration–regeneration followed by a rise in inflammation and fibrosis and exhaustion of the satellite cell population [9,10, reviewed in 11]. For example, in a 9-year-old DMD patient, the proliferative life span of satellite cells was approximately one-third that of an age-matched control [12]. In contrast to DMD which evolves in early childhood, dysferlinopathy is an autosomal recessive late-onset MD with a mutation in the *dysferlin* gene that appears in patients between the ages of 20 to 30 years [13,14]. Though not characterized by the aggressive degeneration–regeneration cycles of DMD, similar cycles have been shown to occur along with a significant rise in inflammation and fibrosis [15–17].

Apoptosis has been shown to increase and become a leading cause of myofiber degradation following necrosis [reviewed in 18, 19] under acute stress such as electrical stimulation [20], in chronic conditions such as cachexia [21], in aging [22], and in MDs [11,23]. In *mdx* mice and DMD patients, upregulation of pro-apoptotic

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CMD, congenital MD; DMD, Duchenne MD; DMEM, Dulbecco's Modified Eagle's Medium; ERK, extracellular signal-regulated protein kinase; DAPI, 4′,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; MDs, muscular dystrophies; NO, nitric oxide; PI3K, phosphoinositide 3 kinase; PPH3, phospho-histone H3; TGFβ, transforming growth factor β; YY1, Ying-Yang 1.

* Corresponding author at: Dept. of Animal Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel.
E-mail address: orna.halevy@mail.huji.ac.il (O. Halevy).

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proteins such as Bax and caspases has been observed in myofibers, suggesting that under pathological conditions, these myofibers undergo apoptosis [11]. Detection of apoptosis and decreased Bcl2 expression have been observed in patients with other MDs, such as Limb Girdle MD type 2C [24] and congenital MD (CMD) type 1A [25]. In agreement with others [11,19,21], we recently reported that in the *mdx* mouse model of DMD, the diaphragm, which is the muscle that is most affected by this disease, the number of apoptotic satellite cells and macrophages is higher than in wild-type mice [26]. In contrast, myofibroblasts, the activated form of fibroblasts, become resistant to apoptosis in dystrophic muscles [26–28]. Thus far, to the best of our knowledge, the presence of apoptosis has not been investigated in dysferlinopathies.

Halofuginone, an inhibitor of Smad3 phosphorylation downstream of the transforming growth factor β (TGFβ) signaling pathway, results in inhibition of the fibroblast-to-myofibroblast transition and fibrosis [29], reviewed in [30], and prolyl-tRNA synthetase activity results in inhibition of Th17 cell differentiation, thereby inhibiting inflammation [31,32]. Halofuginone has been reported to improve muscle histopathology in mouse models with early disease onset, such as *mdx* and the laminin α2-deficient dy^{2J}/dy^{2J} mouse model of CMD [33,34], reviewed in [35]. More recently, it has been reported that halofuginone also improves muscle histopathology and function in a dysferlin-knockout mouse model, through a direct effect on muscle cells. It promotes myotube fusion of primary myoblasts derived from normal and dystrophic muscles [36], and inhibits apoptosis of satellite cells and myofibers in the *mdx* mouse muscle [26]. Halofuginone promotes the phosphorylation of Akt and mitogen-activated protein kinase (MAPK) family members, and enhances the association of phosphorylated Akt and MAPK/extracellular signal-regulated protein kinase (MAPK/ERK) with the nonphosphorylated form of Smad3, resulting in decreased Smad3 phosphorylation [36]. Both MAPK/ERK and phosphoinositide 3 kinase (PI3K)/Akt pathways are involved in the myogenic lineage; the MAPK/ERK pathway has been reported to be mainly involved in early stages of myoblast proliferation [37,38], while the PI3K/Akt pathway has been shown to be crucial for later stages of their terminal differentiation and for cell survival [39–41].

The promotive effect of halofuginone on the MAPK/ERK pathway prompted us to look into its effect on cell-cycle events of satellite cells in mouse models of dysferlinopathy and DMD. Halofuginone promoted the entrance of single-myofiber-attached satellite cells into the cell cycle via the MAPK/ERK pathway. Moreover, the increased apoptosis in myofibers and myoblasts of the dysferlin-deficient mouse model was reduced by halofuginone treatment via the PI3K/Akt pathway.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), sera and antibiotic-antimycotic solution were purchased from Biological Industries (Beit-Haemek, Israel). Ly294002 and UO127 were purchased from Calbiochem (Gibbstown, NJ). Halofuginone bromohydrate was obtained from Akashi Therapeutics, LLC (Newton, MA).

2.2. Mice

Male *dysf* $^{-/-}$ [mixed 129SvJ and C57/BL/g background (Stock 006,830) in which a 12-kb region of the *dysf* gene containing the last three exons is deleted, removing the transmembrane domain], *mdx* [C57BL/10ScSn-Dmd^{mdx}/J (Stock 001,801), dystrophin-deficient)] and C57/Bl/6 J (termed Wt or C57) mice (Jackson Laboratories, Bar Harbor, ME) were housed in cages under constant photoperiod (12 L:12 D) with free access to food and water. All animal experiments were carried out according to the guidelines of the Volcani Center Institutional Committee for Care and Use of Laboratory Animals (IL-234/10).

2.3. Cell preparation and maintenance

Primary myoblasts from the hind-leg muscles of 6-week-old mice were prepared as described previously [42]. Cells were plated at a low density of 3 x 10^5 in Petri dishes (90 mm diameter) to avoid spontaneous differentiation and grown at similar rate in DMEM supplemented with 20% (*v*/*v*) fetal calf serum (FCS) at 37.5 °C with humidified atmosphere and 5% CO_2 in air.

2.4. Single myofiber preparation and immunostaining

Single myofibers were isolated from the gastrocnemius muscle as described previously [26]. Briefly, six mice were sacrificed and the gastrocnemius muscles (6–7 muscles) were carefully removed. The outer connective tissue was removed and groups of three muscles were immersed in a 2.5-ml solution of 0.28% (*w*/*v*) collagenase type I in DMEM for 60 min for Wt and *mdx* mice, or for 90 min for *dysf* $^{-/-}$ mice. The collagenase-treated muscle was then transferred to horse serum (HS)-coated Petri dishes containing 10 ml of DMEM with 10% (*v*/*v*) HS for full coverage of the digested fibers, and triturated with a wide-mouth pipette. Myofibers were then washed three times with 10 ml DMEM with 10% HS and placed in 90-mm gelatin-coated plates and remained floating. Trypan blue staining revealed that almost 100% of these myofibers were viable (data not shown). For immunostaining, the myofibers were transferred to 35-mm plates, fixed with 4% paraformaldehyde and then incubated with Triton X-100 (0.5% *v*/*v* in PBS) and blocked with 20% (*v*/*v*) goat serum (GS) in PBS. The myofibers were incubated overnight at 4 °C with the following polyclonal antibodies: anti-Bax (1:150, Santa Cruz Biotechnology), anti-Bcl2 (1:150, Calbiochem), anti-phospho-histone H3 (PHH3), anti-phospho-Akt and anti-phospho-p42/44 (each at 1:1000 dilution, Cell Signaling, Beverly, MA), and monoclonal anti-MyoD (1:150, Santa Cruz Biotechnology), followed by incubation with Alexa 594 goat anti-rabbit IgG or Alexa 488 goat anti-rabbit IgG (1:300, Jackson Laboratories) secondary antibody for 1 h at room temperature. Nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO) in PBS. The myofibers were visualized under a fluorescence microscope (Olympus, Hamburg, Germany) with a DP-11 digital camera (Olympus). Negative control staining without the first antibody revealed some autofluorescence of the myofiber, but with no nucleus staining (data not shown).

2.5. 5-Bromo-2′-deoxyuridine (BrdU) incorporation

Single myofibers were cultured for 6 h in DMEM containing 10 μM BrdU (Sigma) and immunostained with mouse anti-BrdU antibody (G3G4, 1:2000, Becton Dickinson). Cultures were fixed in 2% paraformaldehyde for 15 min and then incubated with Triton X-100 (0.5% in PBS) and blocked with 5% GS and 1% HS in PBS. The cultures were incubated in 2 N HCl/0.2% Triton X-100 in double-distilled water for 10 min at room temperature followed by 5 min in 50 nM glycine in PBS. Incubation of myofibers with mouse anti-BrdU antibody was followed by incubation with biotinylated donkey anti-mouse IgG (1:250, Jackson Laboratories) for 1 h and in Texas Red streptavidin (1:300, Jackson Laboratories) for 30 min. Nuclei were stained with DAPI.

2.6. Western blot analysis

Western blot analysis was performed as described previously [43]. Briefly, equal amounts of protein (30 μg or 40 μg for myoblasts or