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α-Cyclodextrin enhances myoblast fusion and muscle differentiation by the release of IL-4

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ABSTRACT

Muscle fibers are formed during embryonic development by the fusion of mononucleated myoblasts. The spatial structure and molecular composition of the sarcolemma are crucial for the myoblast recognition and fusion steps. Cyclodextrins are a group of substances that have the ability to solubilize lipids through the formation of molecular inclusion complexes. Previously, we have shown that methyl-β-cyclodextrin (MbCD) enhances muscle differentiation. Here, we analyzed the effects of α-cyclodextrin (aCD) during myogenesis. Myogenic cultures treated with aCD showed an increase in myoblast fusion and in the expression of myogenin, sarcomeric tropomyosin and desmin. aCD-conditioned media accelerates myogenesis in a similar way as aCD does, and increased levels of IL-4 were found in aCD-conditioned media. aCD-induced effects on myogenesis were inhibited by an anti-IL4 antibody. These results show that α-cyclodextrin induces myogenic differentiation by the release of IL-4.

1. Introduction

The formation of a skeletal muscle fiber begins with the withdrawal of committed mononucleated precursors from the cell cycle. These myoblasts elongate while aligning with each other, guided by recognition between their membranes. This step is followed by cell adhesion, fusion and the formation of long and striated multinucleated myotubes. In particular, when myoblasts withdraw from the mitotic cycle, significant modifications take place in a number of biochemical and biophysical properties of the plasma membrane, including cell surface proteins, phospholipid distribution across the bilayer, membrane fluidity and remodeling [1]. Many plasma membrane proteins, as well as many soluble proteins, have been implicated in myoblast adhesion and fusion [2–5]. Although cell surface proteins are indispensable to these processes, plasma membrane lipids are also involved. It has been shown that phosphatidylserine is transiently exposed at the sites of cell–cell contact and is functionally required for myotube formation [6]. Furthermore, addition of purified phospholipase C to the culture medium completely inhibits myoblast fusion in a reversible manner without affecting cell proliferation [7]. Membrane cholesterol has also been implicated in myoblast fusion. The addition of cholesterol to the medium 4 h before fusion onset inhibits fusion [8]. It has been shown that a decrease in membrane cholesterol is necessary for myoblast fusion [9–11]. Using filipin and freeze-fracture electron microscopy, Sekiya and collaborator showed that the early stages of fusion were characterized by the depletion of cholesterol from the membrane apposition sites, at which the plasma membranes of two adjacent cells were in close contact. Membrane fusion took place at several points within the filipin–cholesterol complex-free areas.

Although several papers are dedicated to study the involvement of membrane lipids during myoblast fusion, a full comprehension of this complex molecular and cellular phenomenon is still incomplete. One simple approach to study the role of membrane lipids during muscle differentiation is to selectively deplete membrane lipids from myogenic cells. The most common means of modifying the lipid content of cell membranes is the incubation of cells with cyclodextrins (CDs), a family of compounds, which, due to the presence of a relatively hydrophobic cavity, can be used to extract lipids from cell membranes. Different cyclodextrins have different potencies for solubilizing phospholipids and cholesterol: β-cyclodextrins (bCDs) are the most efficient in extracting cholesterol from membranes, while α-cyclodextrins (aCDs), on the other hand, are the most efficient in extracting phospholipids [12]. These differences have been attributed to the size and hydrophobicity of the CD inner cavities [13]. A methylated form of bCD, the methyl-β-cyclodextrin (MbCD), was found to be even more efficient than

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bCD in extracting cholesterol from cell membranes and thus allowing the investigation of cellular and molecular mechanisms associated with cholesterol depletion. Our group has shown that cholesterol depletion by MbCD induces the differentiation of chick-cultured myogenic cells [14] by the activation of the Wnt/β-catenin signaling pathway [15,16].

aCD has been widely used in the literature as an inactive analog control drug for MbCD treatment, since it is structurally similar to MbCD but does not extract cholesterol from cell membranes. The main interest of the present work was to study the effects of aCD during myogenesis. Surprisingly, our data shows that primary cultures of chick myogenic cells treated with aCD showed an increase in myoblast fusion, in myotube thickness, and in the expression of myogenin, tropomyosin and desmin, and therefore is not a suitable control for MbCD treatment in myogenic cells.

2. Materials and methods

2.1. Antibodies and fluorescent probes

DNA-binding probe 4,6-diamidino-2-phenylindole dyhydrochloride (DAPI), Alexa Fluor 488- and Alexa Fluor 546-conjugated goat anti-rabbit and goat anti-mouse immunoglobulin G antibodies were purchased from Molecular Probes (Eugene, OR, USA). The following antibodies were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA): rabbit polyclonal anti-desmin, mouse monoclonal anti-α-tubulin (clone DM 1A), mouse monoclonal anti-sarcomeric tropomyosin (clone CH1) and mouse monoclonal anti-sarcomeric α-actinin (clone EA-53). Mouse monoclonal anti-myogenin antibody (clone F5D) from Developmental Studies Hybridoma Bank (DSHB, University of Iowa, USA) was used. Peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Amersham (USA).

2.2. Primary myogenic cell cultures

All cell culture reagents were purchased from Invitrogen (São Paulo, Brazil). Primary cultures of myogenic cells were prepared from breast muscles of 11-day-old chick embryos [14]. Cells were plated at an initial density of 7.5×10^5 cells/35 mm culture dishes onto 22 mm-aclar plastic coverslips (Pro-Plastics Inc., USA) previously coated with rat tail collagen. Cells were grown in 2 ml of medium (minimum essential medium with the addition of 10% horse serum, 0.5% chick embryo extract, 1% L-glutamine and 1% penicillin–streptomycin) under humidified 5% CO_2 atmosphere at 37 °C. Twenty-four-hour cultures were treated for 30 min with either α-cyclodextrin (aCD; Sigma–Aldrich) or methyl-β-cyclodextrin (MbCD; Sigma–Aldrich), both at a final concentration of 2 mM. The 2 mM final concentration of both aCD and MbCD was chosen for cell culture treatments because our group has previously shown that 2 mM of MbCD is sufficient to induce skeletal and cardiac muscle cell differentiation without interfering with cell viability [14–17]. After treatment, cultures were washed with fresh cultured medium and grown for the next 3, 24 or 48 h.

Phase contrast images of 72 h-myogenic cultures were acquired and the regions containing the larger diameter of each myotube were measured using the public domain software ImageJ (http://rsb.info.nih.gov/ij/). Fifty microscopic fields for each culture condition were used from at least three independent experiments.

Some myogenic cultures (with 24 h) were grown in the presence of one of the following conditioned media: collected from a 3 h untreated culture, collected from a 24 h untreated culture, collected after 3 h of aCD treatment, collected after 24 h of aCD treatment, collected after 3 h of MbCD treatment, or collected after 24 h of MbCD treatment.

Other myogenic cultures (with 24 h) were treated with aCD (2 mM) for 30 min and then grown in the presence of either a mouse monoclonal antibody against IL-4 (final concentration of 10 μg/ml, R&D Systems, USA) or a recombinant IL-4 molecule (final concentration of 10 ηg/ml, R&D Systems, USA) for the next 24 h.

2.3. Lipid extraction and cholesterol determination

Total lipids were extracted from the following conditions of the liquid culture media: minimum essential medium (MEM), growth medium stock solution (GM; prior to cell feeding), growth medium from untreated cell cultures (Ct), growth medium collected immediately after aCD treatment, and growth medium collected immediately after MbCD treatment. Briefly, myogenic cells were washed with cold phosphate buffered saline (PBS) prior to cell detachment and disruption in 4 ml of the extraction solution (chloroform:methanol:HCl, 2:1:0.075, v/v), as previously described [18]. The addition of 0.5 ml of 0.6 N HCl, followed by intense agitation and centrifugation (10 min, 300 *g*) allowed us to isolate the organic phase (containing the lipids). The final organic phase from each sample was dried under N_2 gas and quantified gravimetrically. Cholesterol was determined according to the method described by Courchaine and co-workers [19]. Briefly, the dried lipids from each sample in duplicate were solubilized in 750 μl of acetic acid, followed by the addition of 500 μl of a color reagent (0.05 g $FeCl_3$, 2 ml H_3PO_4, 23 ml H_2SO_4), vigorously homogenized in a vortex mixer, and kept for 10 min at room temperature. The absorbance of each sample was determined in a U-2001 Hitachi spectrophotometer at 550 nm. Cholesterol (Sigma–Aldrich) was used as the standard. The results are expressed as mg of cholesterol per ml of sample. Aliquots (10 μl) from each sample were used for protein determination by the phenol folin reagent, using bovine serum albumin as the standard [20].

2.4. Lipid separation by thin layer chromatography (TLC)

The lipids dried under N_2, were reconstituted in 90 μl of chloroform:methanol:water (7.5:2.5:0.2 v/v), and spotted onto heated-activated silica-gel 60 TLC plates. The plates were developed in chloroform:acetone:methanol:acetic acid:water (120:45:39:36:24, v/v), and the lipids developed by the exposure of the TLC plates to iodine vapors. The identification of each specific phospholipid was obtained by comparison of the relative mobility (R_f values) of commercial standards in the same solvent system.

2.5. Immunofluorescence microscopy and digital image acquisition

Myogenic cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. They were then permeabilized with 0.5% Triton-X 100 in PBS for 30 min. The same solution was used for all subsequent washing steps. Cells were incubated with primary antibodies for 1 h at 37 °C. After incubation, cells were washed for 30 min and incubated with Alexa Fluor 488 and/or Alexa Fluor 546-conjugated secondary antibodies for 1 h at 37 °C. Nuclei were labeled with DAPI (0.1 μg/ml in 0.9% NaCl). Cells were mounted in ProLong Gold antifade reagent (Molecular Probes) and examined with an Axiovert 100 microscope (Carl Zeiss, Germany) by using filters sets that were selective for each fluorochrome wavelength channel. Images were acquired with a C2400i integrated charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan) and an Argus 20 image processor (Hamamatsu). Control experiments with no primary antibodies showed only faint background staining (supplementary material). Live-cultured cells grown on collagen-coated aclar coverslips were examined, and images were acquired by phase contrast microscopy with the same microscope and digital system described