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Dexamethasone induces dysferlin in myoblasts and enhances their myogenic differentiation

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ABSTRACT

Glucocorticoids are beneficial in many muscular dystrophies but they are ineffective in treating dysferlinopathy, a rare muscular dystrophy caused by loss of dysferlin. We sought to understand the molecular basis for this disparity by studying the effects of a glucocorticoid on differentiation of the myoblast cell line, C2C12, and dysferlin-deficient C2C12s. We found that pharmacologic doses of dexamethasone enhanced the myogenic fusion efficiency of C2C12s and increased the induction of dysferlin, along with specific myogenic transcription factors, sarcolemmal and structural proteins. In contrast, the dysferlin-deficient C2C12 cell line demonstrated a reduction in long myotubes and early induction of particular muscle differentiation proteins, most notably, myosin heavy chain. Dexamethasone partially reversed the defect in myogenic fusion in the dysferlin-deficient C2C12 cells. We hypothesize that a key therapeutic benefit of glucocorticoids may be the up-regulation of dysferlin as an important component of glucocorticoid-enhanced myogenic differentiation.

1. Introduction

Loss of function mutations in the dysferlin gene, DYSF, cause limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (MM), and distal anterior compartment myopathy (DACM) collectively known as dysferlinopathy [1–3]. The two main clinical features of these diseases are progressive loss of skeletal muscle and prominent muscle inflammation [4–10]. Age of onset is typically in early adulthood but there is considerable variability in the age of onset and severity of symptoms [11,12]. There is currently no treatment or cure for LGMD2B/MM [5].

Dysferlin is a 237 kDa, calcium binding, C2 domain-containing, transmembrane protein that is highly expressed in skeletal muscle and localized primarily in the sarcolemma [13,14]. Dysferlin-deficient mouse muscle fibers were defective in resealing laser-induced membrane disruption, therefore, it is thought to play a role in mediating the fusion of membrane vesicles to the sarcolemma at sites of membrane damage in muscle cells [15]. Dysferlin was also required for repairing scrape wounding-induced muscle membrane damage [16]. Interestingly, cultured dysferlin-deficient muscle cells from dysferlinopathy patients or normal muscle cells in which dysferlin was knocked down via dysferlin-specific siRNA oligonucleotides demonstrated impaired fusion and differentiation [17]. This and other studies in vitro and in vivo indicated that dysferlin has a role in myogenesis as well as in membrane repair [17–21].

Glucocorticoid treatment is highly effective for inflammatory myopathies and many types of muscular dystrophy such as Duchenne's muscular dystrophy (DMD) and can sometimes cure myositis [22,23]. Therapeutic effects of glucocorticoids are often attributed to their potent anti-inflammatory activity [22]. Studies in DMD patients indicated that glucocorticoid therapy reduced muscle inflammation and muscle proteolysis, while increasing myogenic repair and myoblast proliferation [22,23]. Surprisingly, even though there is significant muscle inflammation in

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LGMD2B/MM, glucocorticoid treatment is not effective in reducing myopathy and in some cases may have resulted in non-recoverable loss of strength [4–10].

Consistent with effects in DMD patients, prednisolone or deflazacort treatment increased muscle strength in *mdx* mice, a dystrophin-null mouse model for DMD [24,25]. The positive effects of prednisone on muscle function were shown to be equivalent in *mdx* compared to the *mdx*;*Rag2*−/− knock out in which B and T lymphocyte development is blocked resulting in immunodeficiency [25]. This indicated that a primary beneficial effect of glucocorticoids may be on the muscle itself and not on suppressing the immune system and inflammation [25].

Glucocorticoids have a broad range of effects in the body that are mediated at the molecular level by direct binding to the glucocorticoid receptor (GR) which activates it as a transcription factor [26–28]. GR is expressed in virtually all tissues, yet it has highly specific dose and context-dependent effects [26–29]. While glucocorticoids are beneficial for treating muscle diseases they also have well-documented catabolic effects on muscle [30]. The mechanisms underlying the positive versus negative effects of glucocorticoids on muscle are not understood but have been shown to be dose and context-specific both in vitro and in vivo [29,30]. For example, the catabolic effects in vivo were most evident under conditions of fasting and were greatly ameliorated by feeding [30]. Previous studies of glucocorticoid effects in vitro on C2C12 myoblasts showed that high doses of the glucocorticoids, dexamethasone or prednisolone, induced cell death and MyoD degradation via the ubiquitin–proteasome pathway [31,32]. On the other hand, treatment with lower doses of dexamethasone or prednisolone resulted in an increase in mRNA levels of the myogenic factors: MyoD, Myf-5, and MRF4 and decreased proliferation, but not death [31]. Treatment of C2C12 cells with IGF-I in combination with dexamethasone resulted in synergistic myogenic differentiation that produced hypertrophic myotubes [33,34]; therefore, glucocorticoids can have direct positive and negative effects on muscle cells.

We sought to understand the molecular basis for why glucocorticoids are ineffective in treating dysferlinopathy patients even though they are beneficial therapy for many other muscular dystrophies and muscle inflammatory diseases [4–10,22,23]. We investigated the effects of glucocorticoid treatment at clinically equivalent doses on the muscle differentiation program in the C2C12 myoblast cell line and in dysferlin-deficient C2C12s in which dysferlin has been knocked down using shRNA [35]. We hypothesized that glucocorticoids may exert part of their therapeutic benefit in muscle diseases by increasing dysferlin levels thereby enhancing myogenic differentiation and muscle repair.

2. Materials and methods

2.1. Cells and reagents

The C2C12-1 murine myoblast cell line was a gift from Drs. A. Miyamoto and G. Weinmaster, Department of Biological Chemistry, UCLA [36]. We named this clone C2C12-1 to distinguish it from the C2C12 P9 clone, the parental C2C12 clone of the dysferlin-deficient C2C12s. Dysferlin-deficient C2C12s were generated using custom designed dysferlin shRNA stable constructs and generously given to us by Michele M. Maxwell, MGH, MA, Robert H. Brown, Department of Neurology, UMass. (Maxwell and Brown, in preparation). C2C12s were maintained in proliferation (growth or PM media: DMEM, 10% fetal bovine serum (FBS) (Omega Scientific), 5% cosmic calf serum (HyClone), 100 U/mL penicillin–streptomycin (Cellgro). Differentiation media (DM) consisted of DMEM, 2% horse serum (Sigma), 100 U/mL penicillin–streptomycin [36]. For dysferlin-deficient C2C12s, 1.5 μg/mL puromycin (Sigma) was added to PM. Dexamethasone (Sigma) was prepared as 10 mM stock in 100% ethanol and diluted in respective media. An equal volume of ethanol was diluted in the respective media as the vehicle control.

2.2. Time course experiment cultures

C2C12 time courses were performed based on the protocol in Doherty et al. [19]. Briefly, cells were plated 1:10 in PM (Day−1: one day before Day 0), media were changed the following day to PM plus 100 nM dexamethasone or 0.001% ethanol as vehicle control (Day 0). Cells were switched to DM on Day 2 for cells harvested on Days 3–7, with subsequent media changes occurring on Days 4 and 6. Media changing and cell harvesting were at the same time each day.

2.3. Myogenic fusion efficiency quantification

Myogenic fusion efficiency was determined as previously described with minor modifications [19]. Briefly, C2C12s were grown on sterile glass coverslips. After 4 days of differentiation (Day 6 of time course), cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton® X-100 (Sigma) in PBS. Cells were blocked in 0.1% Triton® X-100 in PBS, 10% FBS and stained with mouse monoclonal anti-desmin antibody (1:300, Sigma), followed by goat anti-mouse FITC-conjugated secondary antibody (1:500, Jackson ImmunoResearch) and mounted with Vectashield plus DAPI (Vector Labs). Ten fields at 10× magnification per treatment from three separate cultures were captured and analyzed. Using ImageJ, nuclei were counted and classified as being in cells containing one nucleus, two or three nuclei or four or more nuclei. FISH microscope images were captured with Quips mFISH software (Vysis) on a Leica DMR fluorescent microscope. Differences greater than $p < 0.05$ were assumed to be significant.

2.4. Real-time quantitative PCR

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA concentrations were measured using the NanoDrop (Thermo Scientific). cDNA was synthesized with qScript (Quanta BioSciences) using 1 μg of *Turbo* DNase (Applied Biosystems) treated total RNA. Real-time quantitative PCR (qPCR) was carried out using *Power* SYBR Green (Applied Biosystems) on an iCycler iQ5 (Bio-Rad) in triplicate 20 μL reaction volumes using the following procedure: one cycle of 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s. Melting curve data confirmed primer specificity and single product amplification. Dysferlin and myoferlin primers were designed using Vector NTI (Invitrogen) and spanned at least two exons. Primers used were: mouse dysferlin forward 5′-TAT GTG AAA GGC TGG ATG GTG GGA-3′ and reverse 5′-TTG CTC AGC AGG CAG ATA GTC GAA-3′, mouse myoferlin forward 5′-CCC TAC AAA CAG ACC TCC CTG CT-3′ and reverse 5′-CTG AAG GTG GGG TAT AGC CG-3′, mouse MyoD forward 5′-GGA CAG CCG GTG TGC ATT-3′ and reverse 5′-CAC TCC GGA ACC CCA ACA G-3′ [37], mouse myogenin forward 5′-GGA GAA GCG CAG GCT CAA G-3′ and reverse 5′-TTG AGC AGG GTG CTC CTC TT-3′ [37], mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-TGA CGT GCC GCC TGG AGA AA-3′ and reverse 5′-AGT GTA GCC CAA GAT GCC CTT CAG-3′ [38,39], and mouse eukaryotic translation elongation factor 1 epsilon 1 (eEF1ε1) forward 5′-GCG GAG TTG AGG CTG CTG GAG A-3′ and reverse 5′-AGA CTC GGG CCA TTG TTT GTC TG-3′ [39]. Based on geNorm analysis of six candidate reference genes, GAPDH and eEF1ε1 were chosen for normalization using the $2^{-\Delta\Delta C_t}$ method as previously described [40–42]. Effects of experimental