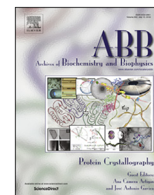
Archives of Biochemistry and Biophysics 603 (2016) 102–109

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi



Dexamethasone rapidly inhibits glucose uptake via non-genomic mechanisms in contracting myotubes

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A R T I C L E   I N F O

Article history:
Received 10 March 2016
Received in revised form
25 May 2016
Accepted 26 May 2016
Available online 28 May 2016

Keywords:
Glucocorticoid
Non-genomic effect
Glucose uptake
C2C12 myotube
Contraction
Glut4 translocation

A B S T R A C T

Glucocorticoids (GCs) are a class of steroid hormones that regulate multiple aspects of glucose homeostasis. In skeletal muscle, it is well established that prolonged GC excess inhibits glucose uptake and utilization through glucocorticoid receptor (GR)-mediated transcriptional changes. However, it remains obscure that whether the rapid non-genomic effects of GC on glucose uptake are involved in acute exercise stress. Therefore, we used electric pulse stimulation (EPS)-evoked contracting myotubes to determine whether the non-genomic actions of GC were involved and its underlying mechanism(s). Pretreatment with dexamethasone (Dex, 10 μM) significantly prevented contraction-stimulated glucose uptake and glucose transporter 4 (Glut4) translocation within 20 min in C2C12 myotubes. Neither GC nuclear receptor antagonist (RU486) nor protein synthesis inhibitor (cycloheximide, Chx) affected the rapid inhibition effects of Dex. AMPK and CaMKII-dependent signaling pathways were associated with the non-genomic effects of Dex. These results provide evidence that GC rapidly suppresses glucose uptake in contracting myotubes via GR-independent non-genomic mechanisms. AMPK and CaMKII-mediated Glut4 translocation may play a critical role in GC-induced rapid inhibition of glucose uptake.
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1. Introduction

Glucocorticoids (GCs) are a class of steroid hormones that regulate multiple aspects of glucose homeostasis. It has been long believed that GCs take those effects mainly via classic genomic mechanisms, which are also largely responsible for GCs' side effects. Specifically, GCs can diffuse freely across plasma membranes and bind to receptor complexes located in the cytoplasm. The hormone–receptor complex then translocates to the nucleus, binds to the glucocorticoid response element (GRE) and thus leads to transactivation or transrepression of these target genes [1,2]. However, GCs can exert some of their effects in a very short time, which do not involve cytoplasmic glucocorticoid receptor (GR) and protein synthesis. These effects cannot be fully explained by the classic genomic mechanisms. Therefore, the new hypothesis suggests that some of GCs's effects are mediated by rapid non-genomic mechanisms [3].

In skeletal muscle, it is well documented that prolonged GC excess inhibits glucose uptake and utilization by antagonizing insulin response, and then results in mitochondrial dysfunction and muscle atrophy [4,5]. Traditionally, those effects of GCs are thought to be propagated through GR-mediated genomic mechanisms. Interestingly, Bowers et al. reported that mice displayed significantly elevated corticosterone concentrations after acute exercise stress [6]. Mutungi et al. revealed that GCs affected maximum force in slow-twitch muscle fibers via a membrane-GR-dependent rapid/non-genomic mechanism [7]. Since skeletal muscle is one of the

Abbreviations: GCs, glucocorticoids; GRE, glucocorticoid response element; GR, glucocorticoid receptor; AMPK, 5′-monophosphate (AMP)-activated protein kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; Dex, dexamethasone; Glut4, glucose transporter 4; EPS, electric pulse stimulation; DMEM, Dulbecco's modified Eagle medium; DM, differentiation medium; 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; RIPA, radio immunoprecipitation assay; NP-40, Nonidet P-40; PMSF, phenylmethanesulfonyl fluoride; TBS, Tris-buffered saline; NIH, National Institutes of Health; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; Veh, vehicle; Ach, acetylcholine; DAPI, 6,4′-diamidino-2-phenylindole; SEM, standard error of the mean; GM, growth medium; Chx, cycloheximide.

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http://dx.doi.org/10.1016/j.abb.2016.05.020
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main organs during acute exercise stress and glucose is the major fuel source for contracting muscle, it is worth to investigate whether non-genomic effects of GC are involved in glucose uptake during skeletal muscle contraction.

The contraction-evoked promotion in glucose transportation is mediated by two main mechanisms: one involves the activation of adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) [8–12] and the other involves the activation of calcium/calmodulin-dependent protein kinase II (CaMKII) [13–15]. The activation of AMPK and CaMKII enhances the translocation of glucose transporter isoform 4 (Glut4) to cell surface and then increases glucose uptake [16].

Using an electrical pulse stimulator device applicable to differentiated myotubes, we primarily aim to investigate: (1) the rapid non-genomic actions of dexamethasone (Dex, a synthetic fluorinated GC) on glucose uptake in contracting C2C12 skeletal muscle cells, and (2) dose-response relationship and its underlying mechanism(s) of these effects. We found that Dex rapidly inhibited glucose uptake via GR-independent non-genomic mechanisms by decreasing Glut4 translocation to plasma membrane in contracting myotubes stimulated by electric pulse stimulation (EPS). The actions of Dex were associated with the inhibition of AMPK and CaMKII-dependent signaling pathways.

2. Materials and methods

2.1. Cell culture

C2C12 mouse myoblast cells were kind gifts of Dr. Hu, State Key Laboratory of Cell Biology, Shanghai Institutes for Biological Sciences, Shanghai, China. Cells were grown in Dulbecco's modified Eagle medium (DMEM, Gibco, NY, USA), supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified incubator with 95% air and 5% CO_2. When grew confluency, cells were differentiated in differentiation medium (DM) containing 5% horse serum (Gibco, NY, USA) and 1% antibiotics. DM was changed every day and experiments were performed 6–7 days after differentiating.

2.2. Electric pulse stimulation (EPS)

Based on previous reports [17,18], we assembled the proposed electrical stimulation system (Supplementary Fig. 1). Differentiated myotubes were electrostimulated in 6-well culture plates (Corning, NY, USA) using platinum electrodes (Gaoss Union, Wuhan, China) and an electric pulse generator (JL-B, Shanghai Jia Long Educational Instrument Factory, Shanghai, China). Each well was equipped with a pair of "L-type" platinum electrodes, which were connected in series to an electric pulse generator. A biphasic rectangular train of pulses with constant current (voltage: 30 V; pulse duration: 11 ms; pulse frequency: 1, 4, 10, 30 Hz) were continuously delivered to the platinum electrodes for 1–15 min in a CO_2 incubator at 37 °C. Those parameters of electrical stimulation were set according to some previous studies [17,19] and our preliminary work.

2.3. Glucose uptake measurement

Glucose uptake was assayed using a fluorescent D-glucose analogue 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG, Invitrogen, CA, USA) as described earlier [20,21] with slight modifications. Briefly, C2C12 myotubes were treated as indicated, and then cells were further incubated in HBSS buffer containing 50 μM 2-NBDG and stimulated by EPS for 15 min at 37 °C. The medium was then washed twice with pre-cold HBSS, followed by lysis in radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Nantong, China) on ice in the dark for 10 min. The cell homogenate was centrifuged at 16,000*g* for 5 min at 4 °C. Supernatants were aspirated into clean 0.5-ml tubes and prepared for protein quantification. Supernatants were transferred into a 96-well flat clear bottom black microplate (Corning, NY, USA), and the fluorescence was determined using Synergy H1 Multi-Mode Reader (BioTek Instruments, VT, USA) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Fluorescence intensity was detected in duplicate and normalized to protein content in every sample.

2.4. Intracellular ATP evaluation

Cells were washed three times with cold PBS after treatment, and then cells were digested with 0.25% trypsin (Gibco, NY, USA) and re-suspended in PBS. The cell pellet was collected after centrifugation at 1500*g* for 5 min. Intracellular ATP concentration was measured using a luciferase–luciferin ATP Assay Kit (Beyotime Biotechnology, Nantong, China) following the manufacturer's instructions. Always keep the sample on ice during experiments. Final results were normalized to the total protein concentrations, which were determined with the BCA assay kit from Beyotime Biotechnology (Nantong, China). Data were presented as relative luminescence units (RLU)/mg protein.

2.5. Membrane preparation

Membrane preparation was performed using a method published previously [22,23] with slight modifications. In brief, differentiated C2C12 cells were washed twice with PBS, scraped into the same buffer and centrifuged at 1000*g* for 5 min. After aspiration of the supernatant PBS, the cells were harvested in buffer A (50 mM Tris, pH 8.0, and 0.5 mM dithiothreitol) containing 0.1% (v/v) Nonidet P-40 (NP-40), 1 mM phenylmethanesulfonyl fluoride (PMSF) protease inhibitor and 10% PhosSTOP phosphatase inhibitor (Roche Molecular Diagnostics, Indianapolis, USA), and homogenated with a homogenizer. Then the samples were further sonicated for three 10-s pulses (30 s inter-pulse intervals) on ice using a probe sonicator. Homogenates were centrifuged at 1000*g* for 10 min at 4 °C, and the precipitates were suspended in NP-40-free buffer A, incubated on ice for 10 min. After centrifuging, the pellets were suspended again in buffer A containing 0.1% (v/v) NP-40, stood on ice for 1 h and centrifuged at 16,000*g* for 20 min at 4 °C to generate membrane fraction (supernatant). All steps were carried out at 4 °C.

2.6. Western blot

The expression and phosphorylation of each protein were analyzed by Western blotting. Briefly, C2C12 myotubes were homogenized in ice-cold RIPA buffer containing 1 mM protease inhibitor PMSF, and 10% PhosSTOP phosphatase inhibitor. The harvested cell lysates or membrane samples (from above) of equal protein were subjected to 10% SDS-PAGE gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore, Temecula, USA). After blocking for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20, the blots were incubated with primary antibodies at 4 °C overnight. Then membranes were washed four times, and incubated with second antibodies for 1.5 h at room temperature. Bands were visualized using Odyssey Infrared Imaging System. Densitometric quantification of protein bands was performed using National Institutes of Health (NIH) Image J software.

Primary antibody anti-β-actin (dilution: 1:2000) was purchased from Sangon Biotechnology (Shanghai, China), and anti-Glut4

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