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Glucocorticoid-induced CREB activation and myostatin expression in C2C12 myotubes involves phosphodiesterase-3/4 signaling

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ABSTRACT

Muscle atrophy in metabolic conditions like chronic kidney disease (CKD) and diabetes are associated with glucocorticoid production, dysfunctional insulin/Akt/FoxO3 signaling and increased myostatin expression. We recently found that CREB, a transcription factor proposed to regulate myostatin expression, is highly phosphorylated in some wasting conditions. Based on a novel Akt-PDE3/4 signaling paradigm, we hypothesized that reduced Akt signaling contributes to CREB activation and myostatin expression. C2C12 myotubes were incubated with dexamethasone (Dex), an atrophy-inducing synthetic glucocorticoid. Akt/CREB signaling and myostatin expression were evaluated by immunoblot and qPCR analyses. Inhibitors of Akt, phosphodiesterase (PDE)-3/4, and protein kinase A (PKA) signaling were used to test our hypothesis. Incubating myotubes with Dex for 3–24 h inhibited Akt phosphorylation and enhanced CREB phosphorylation as well as myostatin mRNA and protein. Inhibition of PI3K/Akt signaling with LY294002 similarly increased CREB phosphorylation. Isobutyl-methylxanthine (IBMX, a pan PDE inhibitor), milrinone (PDE3 inhibitor) and rolipram (PDE4 inhibitor) augmented CREB phosphorylation and myostatin expression. Inhibition of protein kinase A by PKI reverted Dex- or IBMX-induced CREB phosphorylation and myostatin expression. Our study provides evidence supporting a newly identified mechanism by which a glucocorticoid-related reduction in Akt signaling contributes to myostatin expression via CREB activation.

1. Introduction

Skeletal muscle atrophy is a consequence of numerous conditions, such as chronic kidney disease (CKD), sepsis, diabetes, and cancer, that ultimately reduce patients' quality of life and increases their risk of mortality [1]. These conditions are frequently associated with an elevation in glucocorticoids which are one of the body's stress hormones; in other cases, synthetic glucocorticoids are given to patients as part of a disease treatment. Glucocorticoids can directly contribute to muscle atrophy through activation of the glucocorticoid receptor which upregulates the expression of specific genes involved in the atrophy process (i.e., atrogenes). Glucocorticoids also act indirectly by inhibiting signaling pathways like the insulin receptor substrate (IRS)-1/phosphatidylinositol 3 (PI3)-kinase/Akt pathway which serves to suppress various proteolytic systems in muscle [2,3].

Myostatin is a member of the TGF-β superfamily of proteins that is produced by skeletal muscle and released into the circulation where its acts in a paracrine fashion on myofibers and muscle stem cells (i.e., satellite cells) [4]. It binds to the activin receptor type IIB (ActRIIB) on the cell surface, leading to activation of the SMAD2 and SMAD3 transcription factors [5]. Myostatin reduces muscle mass by inhibiting myogenesis, accelerating protein degradation, impairing protein synthesis, decreasing insulin sensitivity, and increasing inflammatory cytokine expression [6–8]. An increase in the level of the myokine has been linked to reduced abundance of muscle structural genes (e.g., myosin heavy chain IIb, troponin I, desmin)

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and myogenic transcription factors (e.g., MyoD, myogenin). Myostatin gene expression is regulated transcriptionally and epigenetically. Its promoter contains potential binding sites for a variety of transcription factors, including the glucocorticoid receptor, FoxOs, peroxisome proliferator-activated receptor gamma (PPAR-γ), CAATT-enhancer binding proteins (C/EBPs), and cAMP response element binding protein (CREB) [9–12]. Although glucocorticoids, acting via their receptor, have been established to induce myostatin transcription, it remains unclear whether other transactivation mechanisms also exist [13].

CREB is a transcription factor that has been proposed to regulate the myostatin gene. CREB activity is regulated by its phosphorylation on Ser-133 which lead to increased association with the histone acetyl-transferase paralogues CREB-binding protein (CBP) and p300 [14,15]. CREB is phosphorylated in response to hormonal stimuli that increase intracellular cAMP production and can be phosphorylated in response to a wide variety of extracellular signals, including growth factors, osmotic stress and ultraviolet irradiation [16–22]. The role of CREB is variable among different atrophy conditions and not well understood. In spinal muscular atrophy, CREB phosphorylation decreased along with Akt phosphorylation [23]. In contrast, daily formoterol treatment, a β-adrenoreceptor agonist, enhanced muscle CSA and protein synthesis while also increasing CREB phosphorylation [24]. In other atrophy conditions, CREB phosphorylation increases concomitant with muscle atrophy. For example, CREB phosphorylation accompanied muscle atrophy in diabetic mice induced with streptozotocin [1] and in dexamethasone-treated, cultured myotubes [25]. Thus, in conditions known to be associated with elevated glucocorticoids, increased CREB phosphorylation is associated with muscle atrophy.

Protein kinase A (PKA), a major effector of cAMP in skeletal muscle, has an important role in muscle metabolism, including the phosphorylation of CREB on Ser-133 [26]. An increase in intracellular cAMP leads to activation of PKA which, in turn, phosphorylates CREB and other proteins [27]. Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and, therefore, reduce PKA activity. Among PDE isoforms, PDE3 and PDE4 are expressed in skeletal muscle and both were reported to be regulated by PI3K-Akt dependent pathways in other cell types [28–31].

In this study we investigated the role of dexamethasone on Akt/PKA signaling and CREB phosphorylation in cultured myotubes. We hypothesized that the well-established glucocorticoid-mediated reduction in Akt activity leads to increased PKA activity and CREB phosphorylation. We further posited that activation of CREB contributes to the induction of myostatin expression. These hypotheses were tested by evaluating CREB and myostatin in cells incubated with dexamethasone with or without inhibitors of Akt, PDEs and PKA. Our results demonstrate that conditions that negatively impact Akt lead to an increase in CREB phosphorylation and myostatin expression through a PDEs/cAMP/PKA signaling pathway in muscle cells.

2. Materials and methods

2.1. C2C12 myotubes

Mouse C2C12 myoblasts (American Type Culture Collection, Manassas, VA, USA) were grown and passaged in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) plus antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; invitrogen, Carlsbad, CA, USA). At 95% confluence, cells were induced to differentiate into myotubes by replacing the growth media with DMEM containing 4.5 g/L glucose and supplemented with 2% horse serum (Invitrogen) and antibiotics for 3 days before treatments with the media changed daily. Cells were differentiated for at least 4 days to form mature myotubes [32].

2.2. General experimental design

Water-soluble dexamethasone (Dex) from Sigma Aldrich (St. Louis, MO) was dissolved in water and added to C2C12 myotubes at a final concentration of 100 nM. Dex treatments were initiated starting on Day 5 for 24 h incubations and on Day 6 for shorter (≤12 h) incubations; all cells, control and treatment, were harvested on Day 6. All control cells were incubated with an equal volume of vehicle only. Milrinone was purchased from Selleckchem (Houston, TX, USA); Rolipram and 3-isobutyl-1-methylxanthine (IBMX) were from Cayman Chemical (Ann Arbor, MI, USA); LY294002 and H89 were from Sigma Aldrich (St. Louis, MO, USA); PKI 14–22 amide was from Tocris (Minneapolis, MN, USA). All compounds except Dex were dissolved in DMSO and added to cells at final concentrations of 10 μM for milrinone, 25 μM for rolipram, 250 μM for IBMX, 25 μM for LY294002, 10 μM for H89, and 10 μM for PKI 14–22 amide; the treatment times are indicated in the figure legends. When inhibitors were co-incubated with dexamethasone, they were added 15 min prior to Dex treatment.

2.3. Immunoblot analyses

Cells were lysed in a buffer consisting of 0.5 M HEPES (pH 7.4), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA (pH 8.0), 10% glycerol, 10% NP-40, 2 mM Na_3VO_4, 10 mg/ml aprotinin, 5 mg/ml leupeptin, 10 mM benzamidine and 2 mM phenylmethylsufonylfluoride (PMSF). The protein concentration of clarified supernatants was determined using the DC protein assay kit (BioRad Laboratories, Hercules, CA, USA). Proteins were separated by reducing SDS-PAGE and transferred to nitrocellulose membranes which were stained with Ponceau-S to verify that equal amounts of sample proteins were loaded and transferred. The protein blots were incubated overnight with primary antibodies against phospho-CREB (#9198S), phospho-Akt (#9271S), CREB (#9197S), Akt (#9272S) (Cell Signaling, Danvers, MA, USA) in a standard milk-containing blocking solution. Other antibodies used were GDF8/myostatin antibodies (#ab203076) purchased from Abcam (Cambridge, MA, USA) and β-actin antibodies (#sc-47778) purchased from Santa-Cruz Biotechnology (Santa Cruz, CA, USA). Detected proteins were visualized using chemiluminescence methods according to the manufacturer's protocol (BioRad Laboratories, Hercules, CA, USA).

2.4. RNA isolation and Real-Time PCR

RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using the Superscript III First-Strand Synthesis kit (Invitrogen) according to manufacturers' instructions. All RT-PCR reactions were performed with a BioRad CFX96 Real-Time PCR detection system using BioRadiQ SYBR Green supermix (BioRad Laboratories, Hercules, CA, USA). Amplicon authenticity was confirmed by melt curve analysis. Sequence of primers were as follows for GAPDH (forward) 5′-TGGAAAGCTGTGGCGTGAT-3′, (reverse) 5′-TGCTTCACCACCTTCTTGAT-3'; for myostatin (forward) 5′-AGTGGATCTAAATGAGGGCAGT-3′, (reverse) 5′-GGAGTACCTCGTGTTTTGTCTC-3'; GAPDH was used as the normalization control. The parameters of the polymerase chain reaction (PCR) were: 3 min of pre-denaturation at 95 °C, followed by 50 cycles of 10 s at 95 °C and 30 s at 55 °C. The data were analyzed for fold change (ΔΔCt) as previously described [33].

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