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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. 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.

**Keywords**

Glucocorticoid; Myostatin; CREB; Skeletal muscle; Gene expression regulation; Insulin resistance

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 it 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) 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 mg/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 (≤2 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 MgCl₂, 1 mM CaCl₂, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA (pH 8.0), 10% glycerol, 10% NP-40, 2 mM Na₃VO₄, 10 mg/ml aprotinin, 5 mg/ml leupeptin, 10 mM benzamide and 2 mM phenylmethylsulfonylfluoride (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 b-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’-TGAAAGCTGTGGCGTGAT-3’, (reverse) 5’-TGCTTCACCACCTTCTTGAT-3’; for myostatin (forward) 5’-AGTGGATCTAAATGAGGGCAGT-3’, (reverse) 5’-GGAGTACCTGTTTTGTCTC-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].

2.5. Statistical analyses

Data are presented as the mean ± SEM of multiple experiments with at least 3 replicates per experiment. Differences between two groups were compared using a two-tailed Student’s t-test. Differences between three or more treatments were compared using a two-tailed one-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons. All
3. Results

3.1. Dexamethasone inhibits Akt phosphorylation and induces CREB phosphorylation

Our initial experiments focused on comparing the time courses for the dexamethasone-mediated changes in Akt and CREB phosphorylation. Mature C2C12 myotubes were treated with dexamethasone from 3 h to 24 h respectively. There was a progressive decrease in Akt phosphorylation which continued over the course of 24 h (Fig. 1A). Conversely, CREB phosphorylation was significantly increased within 3 h of addition of dexamethasone to the myotubes and the increase was sustained over 24 h (Fig. 1B). Thus, the time courses for dexamethasone-mediated changes in the phosphorylation status of Akt and CREB suggests an inverse relationship between their activities.

3.2. Inhibiting PI3K/Akt induces CREB phosphorylation and myostatin expression

We next examined how inhibition of Akt affects CREB phosphorylation and myostatin expression. C2C12 myotubes were incubated with dexamethasone or LY294002 which attenuates Akt activity by inhibiting phosphatidylinositol-3-kinase (PI3K). After 6h, LY294002 mimicked dexamethasone by increasing CREB phosphorylation and no greater effect was achieved by combining LY294002 plus Dex compared to LY294002 alone (Fig. 1C). Under the same conditions, both Dex and LY294002 increased myostatin mRNA expression; the combination of Dex plus LY294002 did not increase myostatin mRNA more than LY294002 alone (Fig. 1D).

3.3. Inhibiting PDE3/4 and PDEs induce CREB phosphorylation and myostatin expression

In some cell types, Akt activates specific isoforms of PDE which catalyze the hydrolysis of cAMP [28–31]. PDE3 and PDE4 are expressed in skeletal muscle and their activities are influenced by Akt in other cell types [29,31]. In light of this potential Akt-PDE interaction and the opposing actions of dexamethasone on CREB and Akt, we hypothesized that dexamethasone might be activating CREB by inhibiting Akt and PDEs. Hence, we examined the effects of a non-selective pan PDE inhibitor, IBMX, or selective inhibitors of PDE3 (milrinone) and PDE4 (rolipram) on CREB phosphorylation in muscle cells. All three inhibitors increased CREB phosphorylation more than dexamethasone (6 h) (Fig.2A).

According to a previous report, the human myostatin promoter contains potential cAMP response elements to which CREB binds [34]. To evaluate whether activation of CREB through inhibition of PDE3 or PDE4 might contribute to the myostatin-inducing action of dexamethasone, we incubated C2C12 myotubes with dexamethasone, milrinone, rolipram or IBMX for 6h and evaluated myostatin expression. All treatments increased myostatin mRNA (Fig. 2B) and protein levels (Fig. 2C), relative to the levels in control cells. Thus, inhibition of PDE3 or PDE4 is sufficient to increase both CREB phosphorylation and myostatin expression.
3.4. Inhibiting PKA decreases Dex and IBMX-induced CREB phosphorylation

Based on the Akt and CREB phosphorylation time courses and the results of the PDE inhibitor experiments, we reasoned that PKA is the kinase most likely to phosphorylate (i.e., activate) CREB in response to dexamethasone. To test this possibility, we incubated myotubes with either dexamethasone or IBMX combined with H89 or PKI 14–22 amide, two specific inhibitors of PKA. H89 significantly reduced CREB phosphorylation in cells treated for 6 h with either dexamethasone or IBMX (Fig. 3A). Similarly, PKI 14–22 inhibited the induction of CREB phosphorylation by dexamethasone and IBMX as well as blocked the increase in myostatin mRNA by dexamethasone (Fig. 3B and C). These results indicate that PKA mediates the dexamethasone-induced activation of CREB and contributes to the glucocorticoid’s action on myostatin expression.

4. Discussion

We report that dexamethasone induces CREB phosphorylation and myostatin expression in C2C12 myotubes through a signaling pathway that includes Akt, PDE3/4 and PKA in skeletal muscle (Fig. 4). Akt activation via its phosphorylation has been well established to suppress proteolytic systems by phosphorylating the Forkhead box O (FoxO) family of transcription factors (i.e., FoxO1, FoxO3 and FoxO4 in skeletal muscle), a modification that prevents them from translocating to the nucleus and increasing transcription of components of the ubiquitin-proteasome system (e.g., the E3 ubiquitin ligases MuRF1/Trim63 and MAFbx/atrogen-1) and the autophagy-lysosome system (e.g., cathepsin L, BNIP3) [33,35–38]. Although the myostatin promoter is a direct target of the glucocorticoid receptor, and perhaps the FoxOs as well, our data supports a model whereby decreased Akt phosphorylation (e.g., insulin resistance) is another contributor to the induction of myostatin through reduced PDE3 and PDE4 signaling and the resulting changes in the activation state of CREB in skeletal muscle [10,11].

In our study, glucocorticoids caused opposing actions on Akt and CREB phosphorylation, with similar time courses. To elucidate the role of Akt in the phosphorylation of CREB, we inhibited Akt signaling in myotubes using LY294002 or Dex; both treatments increased CREB phosphorylation. PKA is the major kinase that phosphorylates CREB in skeletal muscle [26]. PDEs, specifically PDE3 and PDE4, reduce PKA activity and are regulated by Akt-dependent pathways in non-muscle cell types [28–31]. However, no previous reports have linked Akt/PDEs/cAMP/PKA signaling to either the phosphorylation of CREB or myostatin expression in muscle cells, including models of muscle atrophy. Hence, our findings that inhibition of Akt and PDE3/4 lead to increases in both phospho-CREB and myostatin expression identify a new novel pathway by which glucocorticoids contribute to muscle atrophy via the production of this myokine. Although we did not directly test whether CREB phosphorylation modulates myostatin expression, a previous report that IBMX increases myostatin promoter activity is consistent with our findings [39]. This conclusion is further supported by our finding that an inhibitor of PKA (i.e., PKI 14–22 amide) attenuated the increase in myostatin mRNA by Dex.

Myostatin plays an important regulatory role in muscle atrophy associated with chronic diseases like diabetes, cancer and kidney failure. In many cases, glucocorticoids are a critical
mediator of the atrophy responses [40]. Reduction of myostatin signaling improves insulin signaling, reduces the levels of atrogenes [41] and other components of the ubiquitin-proteasome and autophagy proteolytic systems [42], and improves muscle satellite cell function. Our findings underscore the multi-faceted manner in which myostatin is regulated. They also broaden our knowledge of how PDEs influence muscle mass via modulation of myostatin production and are relevant to patients who are treated with PDE3/4 inhibitors for life-threatening conditions like heart failure; many of these patients exhibit symptoms of muscle atrophy [43].

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Fig. 1. Dexamethasone inhibits Akt phosphorylation and induces CREB phosphorylation. Inhibiting PI3K/Akt induce CREB phosphorylation and myostatin mRNA up-regulation. Mouse C2C12 myotubes were differentiated for 4 days and treated with dexamethasone (Dex, 100 nM) for the indicated times. LY294002 (LY, 25 μM) was used as the PI3K inhibitor. (A) Dex significantly decreased the ratio of pAkt/total Akt from 3 h to 24 h (B) The ratio of pCREB/CREB was elevated after 3 h and remained elevated for up to 24 h. (C) LY increased the ratio of pCREB:CREB vs untreated control cells; treatment with LY and Dex increased the amount of pCREB over Dex alone in C2C12 myotubes. Representative immunoblot results are included. (D) LY, either alone or in combination with Dex, increased myostatin mRNA significantly vs untreated control myotubes. Data are expressed as the mean value ± SE of the arbitrary densitometric units (A.U.) of untreated control values from at least three experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs untreated control myotubes (n = 5 experiments with 2 replicates per experiment for A; n = 3 experiments with 2 replicates per experiment for B; n = 3 experiments with 2 replicates per experiment for C and D). Results of a typical immunoblot are shown above each graph including an α-tubulin blot as a control.
Fig. 2. Inhibiting PDE3 or PDE4 induce CREB phosphorylation and myostatin expression.
C2C12 myotubes were treated with the PDE3 inhibitor milrinone (M, 10 μM), PDE4 inhibitor rolipram (R, 25 μM), pan PDE inhibitor IBMX (I, 250 μM), or PKA inhibitor H89 (H, 10 μM), for 6h. M, R and IBMX significantly increased the pCREB:CREB ratio (A), myostatin mRNA (B), and myostatin protein (C); H89 attenuated these responses. Data are expressed as the mean value ± SE of the arbitrary densitometric units (A.U.) or percentage ± SE of untreated control values from at least three experiments. **P < 0.01, ***P < 0.001 vs control. (n = 3 experiments with 3 replicates per experiment for A, B and C). Representative immunoblot results are shown above the graphs in Panels A and C.
Fig. 3. Inhibitors of PKA attenuate the Dex-induced increase in CREB phosphorylation and myostatin expression.

Inhibition of PKA with H89 (A) or PKI-14–22 amide (B) prevented the increase in CREB phosphorylation induced by Dex or IBMX. PKI-14–22 amide also prevented the induction of myostatin mRNA by Dex (C). Data are expressed as the mean value ± SE of the arbitrary densitometric units (A.U.) or percentage ± SE of untreated control values from at least three experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs control. #P < 0.05, ##P < 0.01, ###P < 0.001 vs Dex alone. ^P < 0.01, ^^P < 0.001 vs IBMX alone. (n = 3 experiments with 2 replicates per experiment).
Fig. 4. Overview of glucocorticoid induction of myostatin.
Glucocorticoids activate their intracellular receptor which inhibits insulin/IRS-1/
phosphatidylinositol-3 kinase (PI3K)/ Akt signaling. This leads to a reduction in PDE3/
PDE4 activity, a resulting increase in cAMP, and activation of PKA. PKA phosphorylates
(i.e., activates) CREB which contributes to increased expression of myostatin mRNA and
protein.