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The cAMP Response Element Binding protein (CREB) is activated by Insulin-like Growth Factor-1 (IGF-1) and regulates *myostatin* gene expression in skeletal myoblast

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ABSTRACT

Myostatin, a member of the Transforming Growth Factor beta (TGF-β) superfamily, plays an important role as a negative regulator of skeletal muscle growth and differentiation. We have previously reported that IGF-1 induces a transient *myostatin* mRNA expression, through the activation of the Nuclear Factor of Activated T cells (NFAT) in an IP_3/calcium-dependent manner. Here we examined the activation of CREB transcription factor as downstream targets of IGF-1 during myoblast differentiation and its role as a regulator of *myostatin* gene expression.

In cultured skeletal myoblast, IGF-1 induced the phosphorylation and transcriptional activation of CREB via IGF-1 Receptor/Phosphatidylinositol 3-Kinase (PI3K)/Phospholipase C gamma (PLC γ), signaling pathways. Also, IGF-1 induced calcium-dependent molecules such as Calmodulin Kinase II (CaMK II), Extracellular signal-regulated Kinases (ERK), Protein Kinase C (PKC). Additionally, we examined *myostatin* mRNA levels and *myostatin* promoter activity in differentiated myoblasts stimulated with IGF-1. We found a significant increase in mRNA contents of *myostatin* and its reporter activity after treatment with IGF-1. The expression of *myostatin* in differentiated myoblast was downregulated by the transfection of siRNA–CREB and by pharmacological inhibitors of the signaling pathways involved in CREB activation. By using pharmacological and genetic approaches together these data demonstrate that IGF-1 regulates the *myostatin* gene expression via CREB transcription factor during muscle cell differentiation.

1. Introduction

Myostatin, or Growth/Differentiation Factor-8 (GDF-8), is a member of superfamily TGF-β and acts as a negative regulator of skeletal muscle growth in vertebrates [1]. In mammals, *myostatin* is expressed primarily in skeletal muscle and acts in an autocrine/paracrine manner to inhibit myoblast proliferation [2,3], differentiation [4–6] and protein synthesis [3]. The biological effects of *myostatin* on muscle cells are mediated through specific binding with the activin type II receptors, subsequently activating by phosphorylation the SMAD signaling pathway (Smad2/Smad3) which act as transcription factors ultimately suppressing myogenesis [7]. In particular, *myostatin* appears to inhibit myoblast differentiation by downregulating the expression of Myogenic Regulatory Factors (MRFs) [4,5] and reducing the IGF-1-induced Akt/TORC1/p70S6K signaling involved in myoblast differentiation and myotube size [8].

In different types of diseases that lead to muscle wasting such as Duchenne Muscular Dystrophy (DMD) or cachexia, *myostatin* is upregulated [9–11]. Moreover, *in vitro* studies have validated that *myostatin* expression is increased in myogenic cells exposed to catabolic conditions such as Dexamethasone [12,13], Reactive Oxygen Species (ROS) [14,15] and Angiotensine II [16] among others. Nevertheless, our group has recently reported that IGF-1, a positive regulator of skeletal muscle growth, induces a transient *myostatin* mRNA expression, through the activation of the NFAT in an IP_3/calcium-dependent manner [17]. This mechanism requires calcium releases from intracellular stores induced by IGF-1, mainly mediated through a PI3K/PLC γ [17].

The aim of this work is to expand the knowledge on IGF-1 induced transcription factors dependent of calcium that are involved in *myostatin* gene expression during myoblast differentiation. We focused on CREB, a transcription factor which plays important roles during differentiation of skeletal muscle cells [18,19]. This transcription factor binds to CREB response elements (CRE) of eukaryotic promoters and its activation can be modulated by calcium

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dependent effectors as Calmodulin Kinase (CaMK) [20,21], Calcineurin (CaN) [20,22], ERK [21,23,24], PKC [25], Protein Kinase B (PKB, Akt) [26] and P38 Mitogen-activated Protein Kinase (P38-MAPK) [21,27]. Moreover, bioinformatics analysis in different vertebrates species show that *myostatin* gene promoters contains conserved CRE boxes, suggesting that *myostatin* may be a transcriptional target of CREB [28,29]. Despite of this, it is unknown whether or not IGF-1 induces *myostatin* expression in a calcium-dependent fashion via CREB in myogenic cells.

2. Materials and methods

2.1. Reagents

Recombinant rat IGF-1 was purchased in R&D System (Minneapolis, MN). Cyclosporin A, U73122, LY294002 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Genistein, Xestospongin C, BimI, Gö-6976 and KN-93 were purchased from Calbiochem (La Jolla, CA). UO126, antibodies against phospho-CREB, CREB, secondary HRP-conjugated anti-rabbit and anti-mouse were obtained from Cell Signaling Technology (Beverly, MA). Antibody against β-Actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CREB-pGL4 y basic pGL4 reporter plasmids were obtained from Promega (Madison, WI). *myostatin* promoter-pGL4 was already reported [17].

2.2. Cell cultures

Primary cultures of skeletal myoblasts were prepared from Sprague-Dawley neonatal hindlimbs as previously reported [30]. Briefly, the muscle tissue was dissected, minced, and treated with collagenase for 15 min at 37 °C. Growth medium was composed of DMEMF-12 (1:1 mixture), 10% bovine serum, 2.5% FBS, 100 U/ml penicillin, and 10 mg/ml streptomycin. At day 3, cultures were differentiated in serum-free medium. The experiments were performed at day 4 when cell were starting to differentiate.

2.3. Western blot analysis

After treatment, cells were solubilized at 4 °C in 30 μl of lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate and a protease inhibitor cocktail (Calbiochem). Proteins extracts were resolved by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Bedford, MA), and blocked for 1 h at room temperature in Tris–buffered saline (TBS), 0.1% Tween 20, and 5% fat-free milk. Incubations with primary antibodies (1:2000) were performed at 4 °C overnight. After incubation for 1 h with HRP-conjugated secondary antibodies (1:2000), membranes were developed by enhanced chemiluminescence (Amersham Biosciences, Amersham, UK). The films were scanned, and the ImageJ program was employed for densitometric analysis of the bands (NIH, USA). To correct for loading, membranes were stripped and blotted against β-Actin (1:4000).

2.4. Cell transfection with luciferase reporter vectors and siRNA–CREB

Primary cells in culture were transiently transfected with Lipofectamine 2000 (Invitrogen). Briefly, 3-day-old myoblasts were transfected with 2 μl Lipofectamine 2000 in 1 ml of DMEM-F12 containing 0.9 μg of the reporter vector DNA and 0.1 μg of the Renilla phRL-TK vector (Promega). CREB mRNA was knocked down using 100 nM of siRNA (sc-72030; Santa Cruz Biotechnology) with 2 μl Lipofectamine 2000 in 1 ml of DMEM-F12. The mixture was maintained for 6 h, media were replaced by serum-media, and cells were maintained until 4 days. Cells were harvested and lysed 9 h after the experiments. Luciferase activity was determined using a dual-luciferase reporter assay system (Promega), and luminescence was measured with a Berthold F12 luminometer.

2.5. Real time PCR (qPCR)

Total RNA was extracted from primary skeletal myoblasts with TRIzol Reagent (Invitrogen), and cDNA was synthesized using

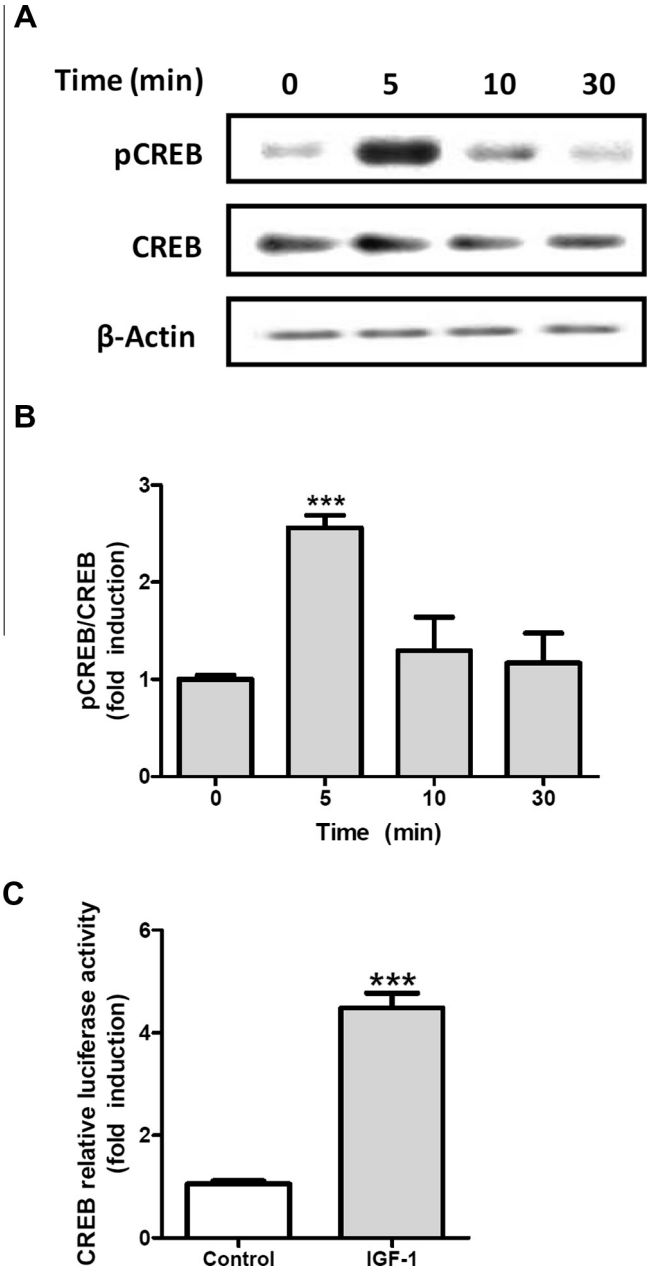

Fig. 1. IGF-1 induces CREB activation in skeletal myoblast. (A) Representative Western blot of phosphorylated CREB, total CREB and β-Actin (loading control). Differentiated myoblasts were stimulated with 10 nM IGF-1 for each indicated times. (B) Densitometric analysis of the Western blot showing pCREB/CREB ratio. (C) Transfected myoblasts with CREB–pGL4 reporter vector stimulated with 10 nM IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. Data are represented as means ± SEM of triplicates from 3 independent experiments and are expressed as fold change relative to values in control cells. Significant differences between IGF-1 treated myogenic cells and control group are shown in $^{***}P < 0.001$.

M-MLV reverse transcriptase and random primers (Invitrogen). cDNA was amplified using *myostatin* primers, and the DNA concentration was normalized against *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*). The *gapdh* primers used were sense 5-CCCCCAATGTATCCGTTGTG-3 (118 bp) and antisense 5-TAGCCCAGGAT GCCCTTTAGT-3 (174 bp) (GenBank: NM_017008.3). The *myostatin* primers were sense 5-GCTCAAACAGCCTGAATCCAAC-3 (2047–2428) and antisense 5-TCACAGTCAAGCCCAAAGTCTC-3 (4497–4518) (GenBank: NC_019151.1). PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The QGene program was used for the analysis of gene expression [31].

3. Results

3.1. IGF-1. induces CREB activation through PI3K/PLC γ/IP3/calcium dependent signaling pathways

To evaluate the effect of IGF-1 in the activation of CREB in skeletal myoblast, cells were stimulated with IGF-1 at physiological concentrations (10 nM). IGF-1 induced a transient phosphorylation of CREB (Fig. 1A and B). At 5 min, post IGF-1 incubation, CREB phosphorylation significantly increased 2.6-fold over control and then decreased to control level remaining basal until the end of the assessment (30 min). Additionally, CREB-dependent transcriptional activity was measured in primary myoblast transfected with a CREB reporter vector. The reporter activity was significantly increased by IGF-1 4.5-fold over control (Fig. 1C). Taking together, these results show that IGF-1 induces activation of CREB and subsequently transcriptional activity dependent of CREB in cultured rat myoblast.

During skeletal muscle differentiation IGF-1 promotes the phosphorylation of IGF-1 Receptor and subsequently PI3 K signaling pathway activation [32]. Therefore, to analyze whether or not IGF-1 induced CREB activation was via IGF-1R/PI3K pathway myoblast were preincubated with pharmacological inhibitors of PI3K (LY294002 or LY, 50 μM) and IGF-1 Receptor (Genistein or Gen, 100 μM). Treatments with LY and Genistein completely inhibited IGF-1-induced CREB phosphorylation (Fig. 2A and C). Moreover, the preincubation of both inhibitors

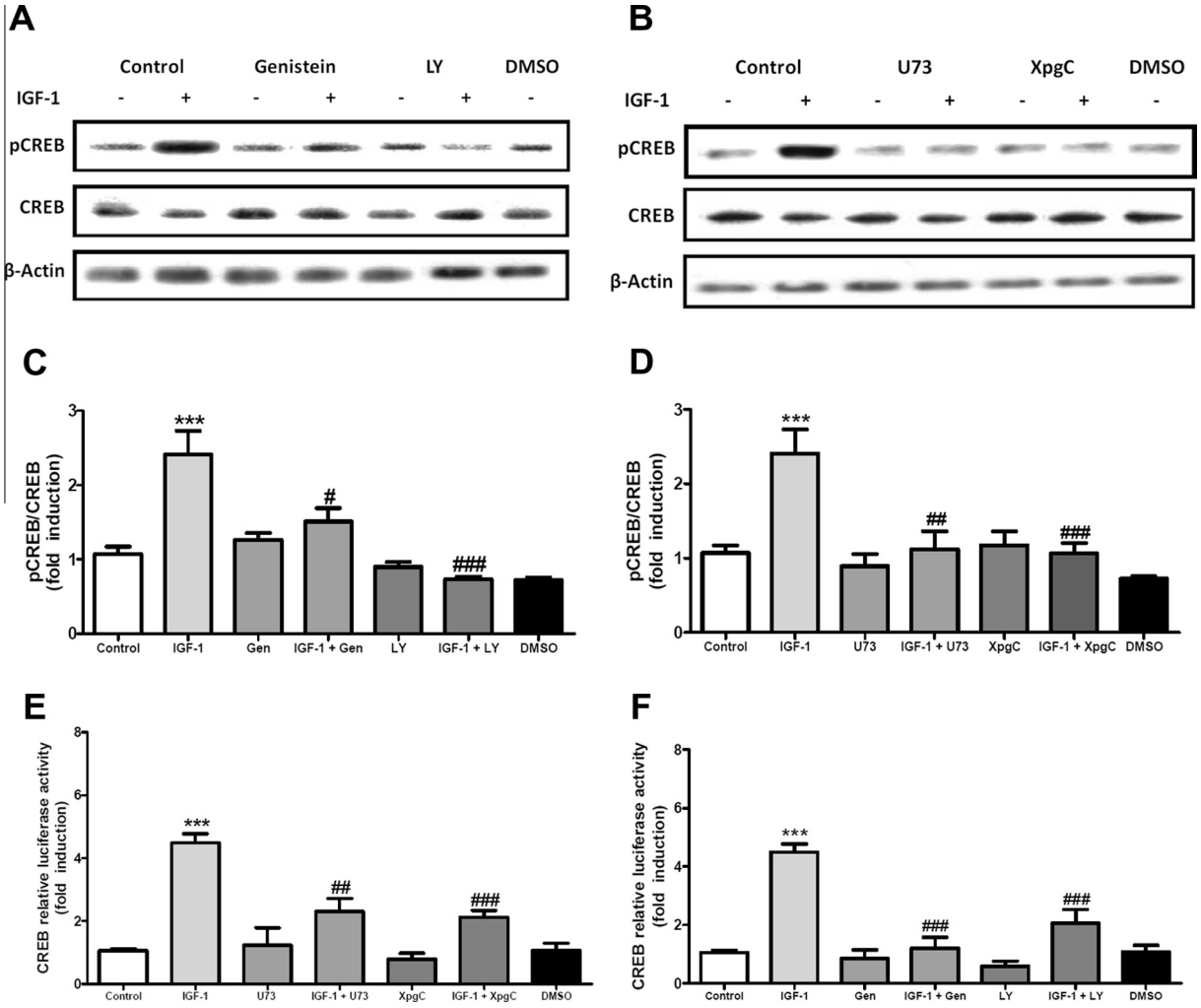

Fig. 2. IGF-1 stimulates CREB activation via IGF-1R/PI3K/PLC pathway in skeletal myoblast. (A–B) Representatives Western blots showing phosphorylated CREB, total CREB and β-Actin (loading control). Differentiated myoblast were incubated with 100 μM Genistein (Gen), 50 μM LY294002 (LY), 50 μM U73122 (U73), 10 μM Xestospongin C (XpgC) or DMSO as solvent control for 30 min, and then stimulated with or without 10 nM of IGF-1. (C–D) Densitometric analysis of Western blot showing pCREB/CREB ratio. (E–F) Transfected myoblasts with CREB–pGL4 reporter vector were pre-incubated with Gen, LY, U73, XpgC or DMSO and then stimulated with or without IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. Data are represented as means ± SEM of triplicates from 3 independent experiments and are expressed as fold change relative to values in control cells. Significant differences between IGF-1 treated myogenic cells and control group are shown in $^{***}P < 0.001$. Differences between IGF-1 + pharmacological inhibitors treated myoblasts and only with IGF-1 are shown in $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

completely blocked the CREB-dependent transcriptional activity induced by IGF-1 (Fig. 2E) suggesting a pivotal role of IGF-1R/PI3K in CREB activation.

We previously reported that IGF-1 induces calcium release from intracellular stores in skeletal myoblast mediated by the activation of the PLC γ/IP_3 signaling pathways [17]. To analyze the contribution of this signaling pathway in CREB activation, myoblast were preincubated with U73122 (U73, 50 μM) and Xestospongin C (XpgC, 10 μM), pharmacological inhibitors of PLC γ and IP_3 Receptor (IP_3R), respectively. In both cases, was observed a significant inhibition of the phosphorylation of CREB and the transcriptional activity induced by this molecule (Fig. 2B, D and F) suggesting an important role of calcium release in CREB activation.

As calcium plays an important role in CREB activation, the contribution of cellular sensors of calcium in subsequent steps of signal transduction were evaluated by Western blot and Reporter vector assays in the presence of pharmacological inhibitors of different signaling pathways. In C2C12 myotubes CaMK II and CaN are required for CREB activation by IGF-1 [20]. We confirmed the participation of CaMK II using a specific pharmacological inhibitor, KN-93 (KN, 20 μM), showing a decrease in CREB phosphorylation as well as CREB reporter activity (Fig. 3A, C and E) However, there was no blockage of CREB activation with Cyclosporin A (CsA, 5 μM), indicating no participation of CaN in CREB activation (Fig. 3B, D and F).

In a previous work, it has been reported that CREB activation is induced by PKC via IP_3R-dependent calcium release in depolarized primary myotubes [25]. Therefore to study the participation of PKC in CREB activation, we exposed cells to BimI (10 μM), a specific inhibitor of most PKC isoforms, and to Gö-6976 (GO, 2 μM), a specific inhibitor of calcium-responsive PKCs. There was a significant decrease in CREB phosphorylation as well as CREB reporter activity in both cases (Fig. 3A, C and E).

In cardiac myocytes IGF-1 stimulates IP_3R-dependent calcium release and ERK which subsequently promotes the activation of CREB [21]. In this context, U0126 (UO, 10 μM), a selective MEK 1/2 inhibitor that blocks the MEK-ERK pathway inhibiting ERK phosphorylation, was employed to determine whether or not ERKs are involved in CREB induction. There was a significant inhibition of phosphorylation and transcriptional activation of CREB by using this inhibitor (Fig. 3B, D and F). Taking together, these results show that IGF-1 induces activation of CREB mediated by the CaMK II, PKC and MEK/ERK.

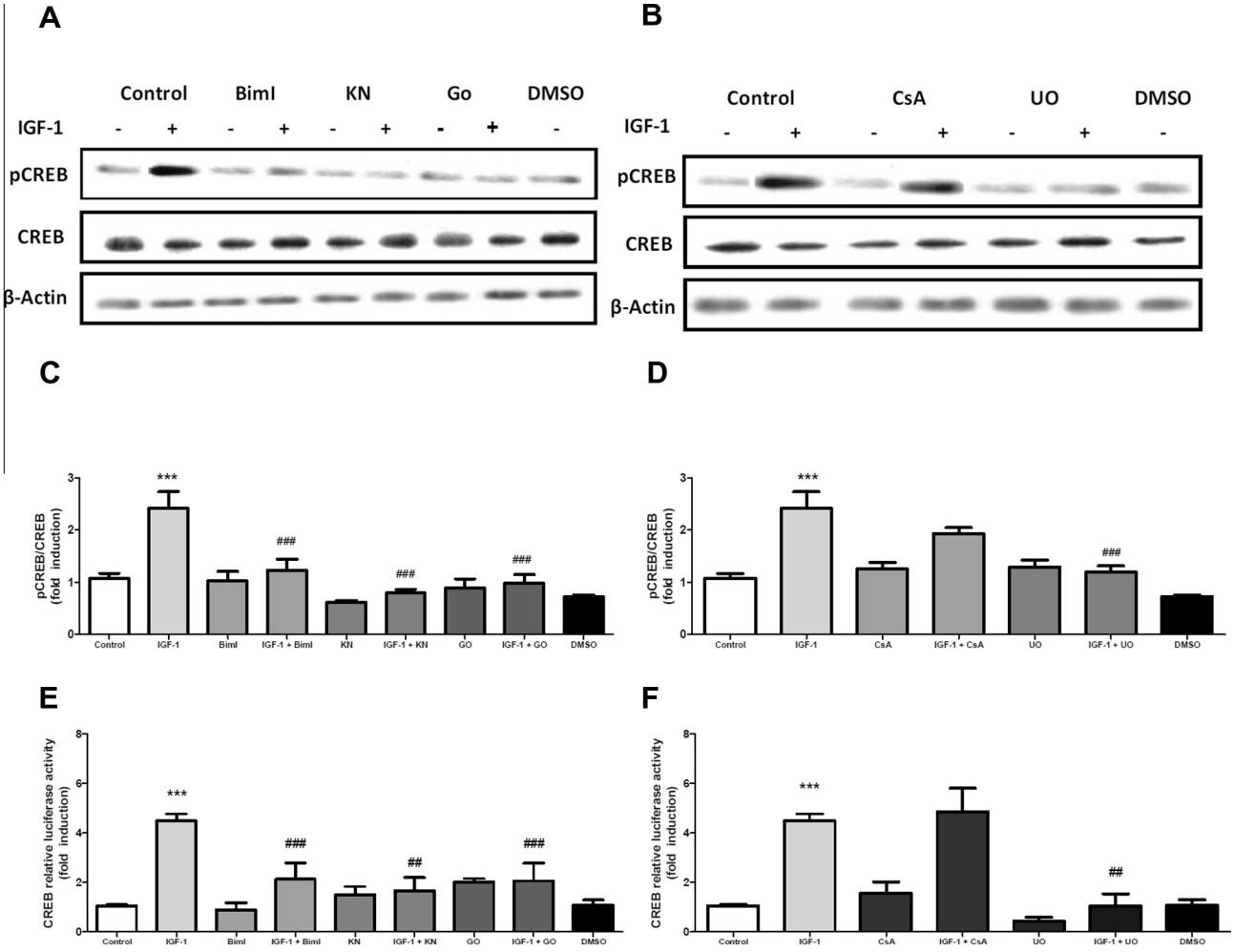

Fig. 3. IGF-1-induced calcium-dependent proteins stimulate CREB activation in skeletal myoblast. (A–B) Representative Western blots showing phosphorylated CREB, total CREB and β-Actin (loading control). Differentiated myoblasts were incubated with 10 μM BimI, 20 μM KN-93 (KN), 2 μM Gö-6976 (GO), 5 μM Cyclosporin A (CsA), 10 μM UO126 (UO) or DMSO as solvent control for 30 min, and then stimulated with or without 10 nM of IGF-1. (C–D) Densitometric analysis of Western blot showing pCREB/CREB ratio. (E–F) Transfected myoblasts with CREB–pGL4 reporter vector were pre-incubated for 30 min with BimI, KN, GO, CsA, UO or DMSO and then stimulated with or without IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. Data are represented as means ± SEM of triplicates from 3 independent experiments and are expressed as fold change relative to values in control cells. Significant differences between IGF-1 treated myogenic cells and control group are shown in $^{***}P < 0.001$. Differences between IGF-1 + pharmacological inhibitors treated myoblasts and only with IGF-1 are shown in $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

3.2. CREB regulates myostatin gene expression

It has been previously reported by our group that *myostatin* mRNA is transiently upregulated following IGF-1 treatment in skeletal myoblasts [17]. Considering that the promoter region of the murine *myostatin* gene contains several candidates of CREB-binding sites, we analyzed *myostatin* mRNA expression and *myostatin* promoter transcriptional activity in myoblast transfected with CREB siRNA. Myoblast incubated with IGF-1 and transfected with CREB siRNA partially blocked *myostatin* expression and *myostatin* promoter transcriptional activity (Fig. 4A and B, respectively), suggesting an important role of CREB in *myostatin* gene expression.

To further analyze the involvement of CaMK II, MEK/ERK and PKC in *myostatin* gene expression, myoblast were pretreated with the inhibitors KN-93 (20 μM), BimI (10 μM), Gö-6976 (2 μM), U0126 (10 μM) and then stimulated with IGF-1. Inhibition of calcium dependent PKC, CaMK II and MEK/ERK resulted in a significant reduction of IGF-1-mediated *myostatin* mRNA expression and *myostatin* promoter activation (Fig. 4C and D, respectively). These results altogether strongly suggest the involvement of calcium dependent signaling pathways in *myostatin* gene expression mediated by CREB.

4. Discussion

The present work gives further evidence of a link between IGF-1 and *myostatin* expression during differentiation of myogenic cells. Particularly, it is highlighted the role of PI3K/PLC γ/IP_3/calcium-dependent signaling pathway the transcription of this muscle growth inhibitor. The CREB activation by IGF-1 mediated by calcium-dependent signaling pathways has been reported in other cellular models. In C2C12 myotubes, IGF-1 significantly increased the levels of phosphorylation of CREB, which is dependent of CaMK II and Calcineurin activity, subsequently leading to regulate the expression of *dihydropyridine receptor* α_{1S} [20]. In cardiac myocytes exposed to hyperosmotic stress, IGF-1 (10 nM) prevents apoptosis inducing the activation of CREB mediated by MEK/ERK, PI3K, P38-MAPK, CaMK II and Calcineurin [21]. In cortical and hippocampal neuronal cells, IGF-1 through a signaling pathway that involves PLC γ, calcium release from IP_3-sensitive internal stores, and CaMK II regulates CREB phosphorylation [33]. However, CREB activation regulated by calcium-dependent signaling pathways is not a general action of IGF-1. This was demonstrated in the rat adrenal medulla, PC12 cells, where IGF-1 promotes the phosphorylation of CREB mediated by MEK/ERK and P38-MAPK, in a PI3K-independent manner [27]. Further evidence was obtained during adipocyte differentiation, where IGF-1 induces the Insulin Receptor Substrate-1 (IRS-1) activation of Ras and ERK 1/2, resulting in phosphorylation of CREB also in a calcium-independent manner [26]. Therefore, our results describe for the first time that in myogenic cells, calcium-dependent signaling pathways induced by IGF-1 are involved in the activation of transcription factor CREB.

Subsequently, and as one of the goals of the present study, the role of IGF-1 and CREB in *myostatin* gene expression, a negative regulator of myogenesis, was examined. We previously

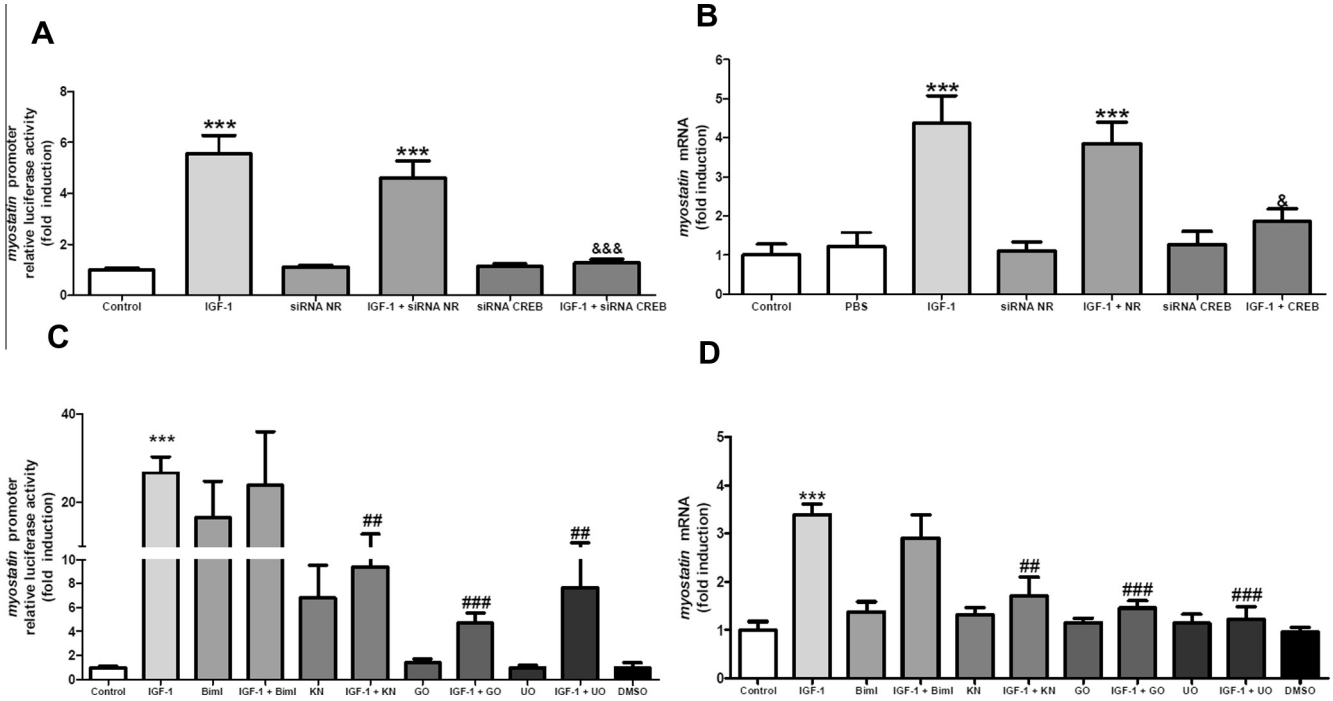

Fig. 4. The transcription factor CREB and IGF-1-activated calcium-dependent proteins regulates *myostatin* gene expression in skeletal myoblast. (A) Co-transfected differentiated myoblasts with *myostatin* promoter–pGL4 reporter vector + 100 nM siRNA CREB or siRNA NR (no related) and then stimulated with or without 10 nM IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. (B) *Myostatin* expression after incubation with siRNA CREB or siRNA NR and then stimulated with or without IGF-1 in differentiated myogenic cells. PBS treated myoblasts are a solvent group control. *Myostatin* mRNA levels were analyzed by qPCR and showed as a relative expression normalized with respect to *gapdh*. (C) Transfected differentiated myoblasts with *myostatin* promoter–pGL4 reporter vector were pre-incubated for 30 min with 10 μM BimI, 20 μM KN-93 (KN), 2 μM Gö-6976 (GO), 10 μM UO126 (UO) or DMSO as solvent control and then stimulated with or without 10 nM of IGF-1. Results were normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase. (D) Myostatin expression after incubation with different inhibitors of calcium-dependent proteins and IGF-1 in differentiated myoblasts. *Myostatin* mRNA levels were analyzed by qPCR. Results showed as a relative expression normalized with respect to *gapdh*. Data are represented as means ± SEM of triplicates from 3 independent experiments and are expressed as fold change relative to values in control cells. Significant differences between IGF-1 treated myogenic cells and control group are shown in $^{***}P < 0.001$. Differences between IGF-1 + siRNA CREB treated myoblasts and IGF-1 + siRNA NR group are shown in $^{\&}P < 0.05$ and $^{\&\&\&}P < 0.001$. Differences between IGF-1 + pharmacological inhibitors treated myoblasts and only with IGF-1 are shown in $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

demonstrated that IGF-1 regulates *myostatin* gene expression through the activation of the NFAT transcription factor in an IP_3/calcium-dependent manner [17]. Therefore, we wondered if other transcription factors activated by IGF-1 could be regulating the expression of *myostatin*. Considering that the 5' promoter region of *myostatin* gene contains putative CRE boxes [28,29], we hypothesize that CREB regulates *myostatin* gene expression. The transfection of myoblast with siRNA–CREB considerably diminished *myostatin* mRNA expression and transcriptional activity of *myostatin* promoter. Complementary the inhibition of calcium dependent signaling pathways CaMK II, PKC and MEK/ERK involved in CREB activation also have a similar effects, suggesting a relevant role of CREB in *myostatin* gene expression. This information supports the involvement of calcium dependent signaling pathways in *myostatin* gene expression.

It is remarkable to note that in skeletal muscle, CREB seems to function as a positive regulator of myogenesis: Biochemical evidence indicates that in differentiating myoblasts, MyoD becomes associated with CREB and is targeted to the retinoblastoma promoter, an essential protein for myoblast cell cycle arrest as well as for the terminal differentiation and survival of postmitotic myocytes [34]. During mouse embryogenesis PKA–CREB is required for Wnt-directed myogenic differentiation [18]. Transgenic mice expressing a dominant-negative CREB transgene in skeletal muscle leads to a dystrophic phenotype associated with a reduced MEF2 activity [35], interestingly a calcium dependent transcription factor involved in *myostatin* mRNA expression. By the other hands, an activated CREB mutant promotes myoblast proliferation and differentiation associated to the expression of *pax7*, *myf5* and *myogenin* [19]. To our knowledge, this report describes for the first time a role of CREB as a negative regulator of myoblast differentiation and complement published data about the regulation of *myostatin* gene expression of calcium-dependent signaling pathways induced by IGF-1 [17]. In this context, we propose that during myoblast differentiation, IGF-1 in physiological doses induces *myostatin* mRNA expression through the activation of calcium-dependent transcription factors such as NFAT and CREB. These results support negative feedback mechanism between positive and negative growth signals controlling muscle fiber size. Whether or not CREB and NFAT are acting synergistically to induce *myostatin* gene expression remained to be determined.

Acknowledgments

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