Creatine increases IGF-I and myogenic regulatory factor mRNA in C_2C_{12} cells

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Abstract Addition of creatine to the differentiation medium of C_2C_{12} cells leads to hypertrophy of the myotubes. To investigate the implication of insulin-like growth factor I (IGF-I) and myogenic regulatory factors (MRFs) in this hypertrophy, their mRNA levels were assessed during the first 72 h of differentiation. Creatine significantly increased the IGF-I mRNA level over the whole investigated period of time, whereas the MRF mRNA levels were only augmented at precise moments, suggesting a general activation mechanism for IGF-I and a specifically regulated mechanism for MRF transcription. Our results suggest therefore that creatine-induced hypertrophy of C_2C_{12} cells is at least partially mediated by overexpression of IGF-I and MRFs.

Key words: Real-time quantitative polymerase chain reaction; Transcription factor; Growth factor; Protein synthesis

1. Introduction

Already in 1972, Ingwall et al. reported that creatine (Cr) stimulated incorporation of labelled precursor into myosin heavy chain, the major myofibrillar protein, and stimulated muscle-specific protein synthesis in both skeletal and cardiac chicken myotubes in culture [1,2]. In another paper, one of Ingwall's co-authors failed to find any regulatory effect of Cr on total protein synthesis in cultured muscle cells [3]. So these early observations have not been confirmed over a 30 year period of time, until recently when it was shown that the fusion of myogenic satellite cells is largely enhanced while creatine monohydrate is added to the culture medium during the differentiation phase [4].

The mechanism(s) by which Cr stimulates muscle protein synthesis remain(s) unknown. Insulin-like growth factor (IGF)-I is a small peptide growth factor similar in structure to pro-insulin which plays a major role in the control of muscle growth [5]. IGF-I is produced by liver, under the control of growth hormone, and is released in blood circulation as an endocrine hormone. Skeletal muscle also produces IGF-I that acts as a paracrine and autocrine growth factor. Overexpression of IGF-I by injection of a plasmid or a viral construct containing IGF-I cDNA into a mouse muscle has been shown to increase muscle mass (15%) and to prevent sarcopenia in old mice [6]. Since the differentiation of myoblasts in culture is stimulated by Cr [4], we hypothesised that IGF-I might be involved in the effect of Cr.

The initiation of muscle differentiation is governed among others by myogenic regulatory factors (MRFs). The MRFs, which include Myo-D, Myf-5, MRF-4 and myogenin, are members of a family of basic helix-loop-helix proteins that act as transcription activators due to their inherent properties as DNA binding proteins. Their binding to DNA initiates the transcription and regulates the expression level of some muscle-specific genes, such as myosin heavy and light chains, α-actin, or creatine kinase [7]. The specific roles of the different MRFs in skeletal muscle differentiation have not yet been completely defined, essentially due to the existence of auto- and cross-regulatory loops between them [8–11]. However, Myo-D and Myf-5 are early factors, mainly involved in myoblast formation and in satellite cell proliferation during regeneration, while myogenin and MRF-4 are late-acting factors, expressed during differentiation [12].

The purpose of this study was to test the role of IGF-I and MRFs in the hypertrophic effect of Cr added to the culture medium of C_2C_{12} cells, during the first 72 h of differentiation.

2. Materials and methods

2.1. Cell culture

The murine C_2C_{12} myoblast cell line (ATCC, Manassas, VA, USA) was propagated in proliferating Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Merelbeke, Belgium) supplemented with 10% foetal bovine serum, penicillin/streptomycin (5000 U/5000 μg/ml) and non-essential amino acids (1%). To induce myogenic differentiation, myoblasts were allowed to grow to approximately 70% confluence and then switched to differentiation medium (DMEM with 2% horse serum, penicillin/streptomycin, 5000 U/5000 μg/ml, and non-essential amino acids 1%). Plates (10 cm diameter, Nunc) were grown in duplicate, and half received Cr (see below) in differentiation medium.

2.2. Protein quantification

To establish the optimal Cr concentration to add in the medium, cells were cultivated with increasing doses of Cr (0, 1, 5, 10 and 20 mM, $n = 3$) in differentiation medium. After 48 h of differentiation, the medium was removed by aspiration and the cells were washed with cooled phosphate-buffered saline (PBS). Myotubes were removed

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Table 1
Primers used for RTQ-PCR. Slope and correlation of standard curve.

Primer name	Sequence (5′ to 3′)	Accession number	Slope	Correlation
β_2-Microglobulin Fw	TGC TAT CCA GAA AAC CCC TCA	NM_009735	−3.37	−0.999
β_2-Microglobulin Rv	GCG GGT GGA ACT GTG TTA CG			
IGF-I Fw	GCT ATG GCT CCA GCA TTC G	NM_010512	−3.39	−0.998
IGF-I Rv	TCC GGA AGC AAC ACT CAT CC			
Myo-D Fw	TCC GTG TTT CGA CTC ACC AG	NM_010860	−3.12	−0.994
Myo-D Rv	AGA AGT GTG CGT GCT CTT CC			
Myf-5 Fw	CTG CCA GTT CTC CCC TTC TG	NM_008656	−2.97	−0.992
Myf-5 Rv	CCG AAG GCT GCT ACT CTT GG			
MRF-4 Fw	GTG GCC AAG TGT TTC GGA TC	NM_008657	−2.88	−0.995
MRF-4 Rv	AAA GGC GCT GAA GAC TGC TG			
Myogenin Fw	TGA GCA TTG TCC AGG CCA G	NM_031189	−3.8	−0.998
Myogenin Rv	GCT TCT CCC TCA GTG TGG CT			

from the plates by addition of 3 ml trypsin (0.5% EDTA, Invitrogen) per plate. After centrifugation, protein pellet was dissolved in 1 ml NaOH 1 N and incubated at 37°C for 12 h. Soluble protein contents of the solutions were determined by spectrophotometric method using a biuret-like reaction (Protein Assay ESL, Boehringer Mannheim kit). After the proliferation, all cells were pooled and then redistributed in several plates. Therefore the number of cells in each plate is believed to be the same and any variation of the protein content can be attributed to the treatment.

2.3. Diameter of myotubes

Five pictures of each plate were recorded. The average diameters of myotubes were measured manually from the cells crossing a diagonal drawn on the picture.

2.4. RNA extraction and real-time quantitative polymerase chain reaction (RTQ-PCR)

Cells were cultured for 0, 24, 48 and 72 h in differentiation medium, with or without 5 mM Cr. At each time point, plates ($n = 4$) were rinsed twice with cooled PBS and total RNA was isolated from cells culture using TRIzol® reagent (Gibco Life Technologies, Paisley, UK) according to the manufacturer's instructions. RNA was quantified by spectrophotometry ($\lambda = 260$ nm) and its concentration adjusted to 0.25 μg/μl using RNase-free water. Reverse transcription using 1 μg of total RNA was done as previously described [13]. RTQ-PCR primers were designed (Primer Express Software, Applied Biosystems) for mouse IGF-I, Myo-D, Myf-5, MRF-4, myogenin, and β_2-microglobulin as housekeeping gene (Table 1). Preliminary experiments of our group showed that β_2-microglobulin mRNA was stable in samples incubated with Cr. RTQ-PCR was carried out using the following cycle parameters: 10 min at 95°C, followed by 40 cycles of 1 min at 60°C and 15 s at 95°C. For each gene, RTQ-PCR was conducted in duplicate with 25 μl reaction volume of 1 ng cDNA. To ensure the quality of the measurements, each plate included for each gene a negative control and a positive control [13]. The threshold cycle (Ct) from a positive sample was used to calculate the inter-assay coefficient of variation (CV). For each gene, the CV was calculated as standard deviation/mean of 2-Ct determined on five different plates and with different mixes. Typically the CV obtained was around 16%.

The IGF-I primers were designed to recognise all the IGF-I isoforms. The mRNA levels were normalised by assigning an arbitrary value of 100% to the mRNA level observed at the beginning of the experiment (0 h).

2.5. Statistics

The effect of Cr was tested by an ANOVA design. Student's *t*-test was used as post hoc. The significance threshold was set to 0.05. The results are presented as mean ± S.E.M.

3. Results

Our results show that adding Cr to the differentiation medium of C_2C_{12} cells induces a general hypertrophy of myotubes (Fig. 1). After 2 days of differentiation, their diameter was 40% larger in the presence of Cr ($P < 0.001$). This hypertrophy was associated with increased protein accumulation. Increasing concentrations of Cr to 10 mM significantly increased protein content ($P < 0.05$) that returned to the control level with 20 mM Cr (Fig. 2). Because 5 mM Cr induced the maximal effect on protein accumulation, the experiments were performed by adding 5 mM Cr to the differentiation medium.

IGF-I mRNA as well as mRNA of the four MRFs (Myo-D, Myf-5, MRF-4 and myogenin) were detectable and quantifiable at each time point we investigated.

In control conditions, the level of IGF-I mRNA exhibited a steady-state level throughout the first 3 days of differentiation

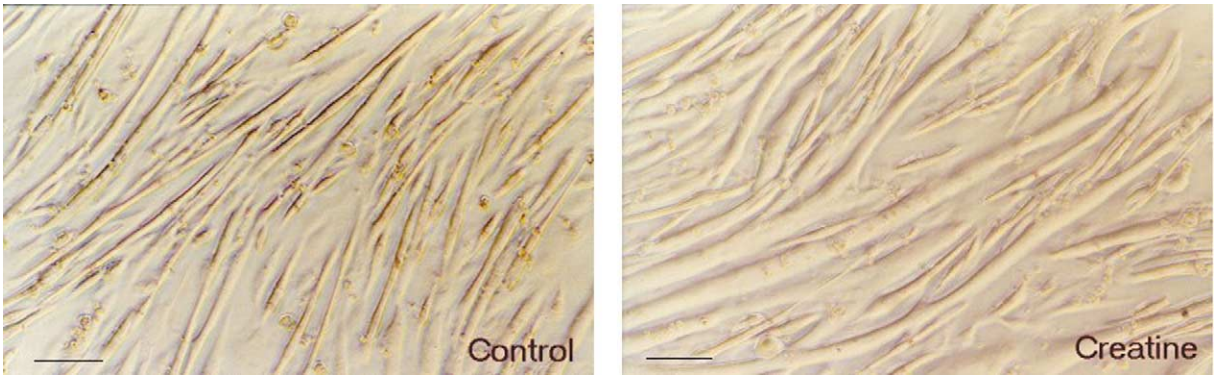

Fig. 1. C_2C_{12} cell hypertrophy induced by Cr. At 70% confluence, C_2C_{12} myoblasts were exposed for 48 h to differentiation medium containing 5 mM Cr. Scale bar, 100 μm.

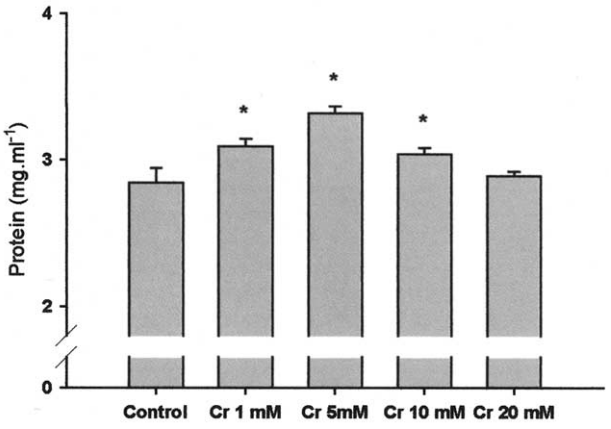

Fig. 2. Dose–response relationship between Cr concentrations and myotube protein content. At 70% confluence, C_2C_{12} myoblasts were exposed for 48 h to differentiation medium containing different concentrations of Cr. After 48 h, cells were collected and cell protein concentrations were assayed as described in Section 2. Results are expressed as mean ± S.E.M. ($n = 3$). *$P < 0.05$ vs Ctrl conditions (Cr 0 mM).

(Fig. 3). However, when cells were incubated with 5 mM Cr, the level of IGF-I mRNA increased progressively to be multiplied by 3.7 after 72 h (ANOVA, $P = 0.041$).

The level of Myo-D mRNA remained stable in control and Cr conditions over the first 48 h of differentiation. Nevertheless, after 72 h, the mRNA level of Myo-D increased up to 150% in control conditions, and to 200% with Cr (ANOVA, $P = 0.001$).

In contrast, Myf-5 mRNA increased in control conditions by 75% one day after cells were placed in differentiation medium and returned to control level the next day. The increase was potentiated by adding Cr in the differentiation medium (ANOVA, $P = 0.040$). After 24 h differentiation, Myf-5 mRNA level reached 230% compared to Ctrl 0 h. Afterwards, it returned to a level similar to control 0 h in both conditions. This means that the reduction in Myf-5 mRNA between 24 h and 48 h of differentiation was faster when C_2C_{12} cells were incubated with Cr.

As soon as the fusion process was initiated, the level of myogenin mRNA increased continuously in control conditions, reaching a 90-fold increase after 72 h of differentiation.

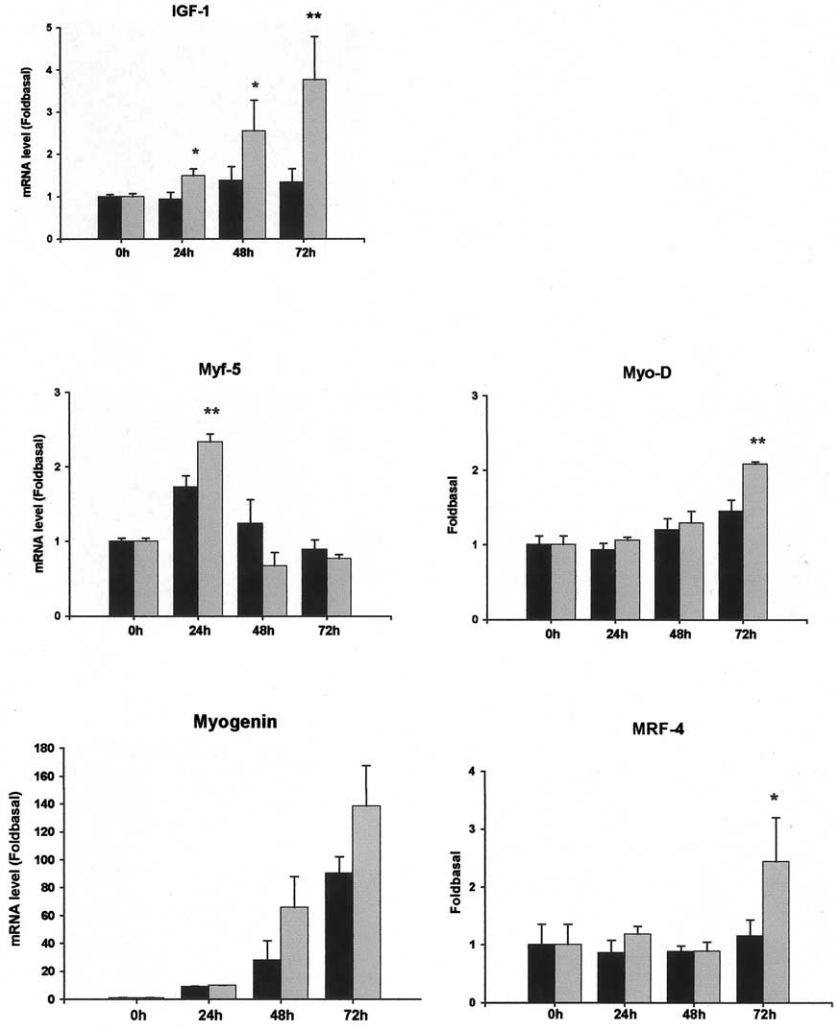

Fig. 3. IGF-I and MRF mRNA expression. At 70% confluence, C_2C_{12} myoblasts were exposed for different periods of time to differentiation medium with (grey bars) or without 5 mM Cr (black bars). After 0, 24, 48 and 72 h of differentiation, cells were collected and gene expression levels for Myf-5, Myo-D, myogenin and MRF-4 were assessed by RTQ-PCR as described in Section 2. A value of 1 was arbitrarily assigned to the control conditions (ctrl 0 h). Results are expressed as mean ± S.E.M. Statistical differences are assessed by Student's t-test: *$P < 0.05$; **$P < 0.01$, Ctrl vs Cr conditions.

This marked induction was even more pronounced in the presence of Cr, reaching a 140-fold increase after 3 days. However, due to the small number of samples and the large variation, the significance threshold was not reached.

Finally, the level of MRF-4 mRNA remained stable during the first 3 days of differentiation. Nevertheless, on the third day, MRF-4 mRNA showed an increase of 230% in the presence of Cr (ANOVA, $P = 0.049$). For all MRFs tested, Cr addition potentiates the induction stimulated by the switching to differentiation medium of C_2C_{12} cells.

4. Discussion

In this study, we investigated the effect of Cr in C_2C_{12} myogenic cells during the first 72 h of differentiation. In the presence of Cr, the average diameter of myotubes was increased suggesting that Cr enhances differentiation of C_2C_{12} cells. The dose–response relationship shows that 5 mM Cr in the medium is the optimal amount. This level of Cr is very high compared with the normal physiological range of concentrations (0.05–0.5 mM). It could elicit various cell responses among which a cellular swelling due to a higher osmotic pressure followed by a regulatory volume decrease [14,15]. The subsequent stretching of membrane could initiate the stimulation of protein synthesis [16]. It is possible that the cell is unable to regulate its volume when larger amounts of Cr are added in the medium (20 mM). This could explain why the protein content was lower in myotubes grown with 20 mM Cr in comparison to those grown with 5 mM Cr, although their diameters were similar.

In any case, our results confirm that Cr stimulates growth and protein accumulation in myogenic cells in culture [1,4]. To assess the role of IGF-I and the MRFs in this trophic effect, we investigated the time course of the induction of their gene expression by Cr.

The sequence of MRF mRNA activation that we observed is similar to the previously reported sequence for MRF protein expression during differentiation [12]. It is commonly accepted that Myo-D and Myf-5 are already highly expressed in undifferentiated myogenic cells before the differentiation programme is activated [17,18] while myogenin and MRF-4 are rather late-acting factors, expressed during the differentiation (Fig. 3). Our results indicate that Myf-5 mRNA still increases during the first 24 h of differentiation whereas the level of Myo-D mRNA remains much more stable showing only a slight increase after 72 h. Moreover, our results show that Cr stimulates the transcription of all MRFs tested. As Dedieu et al. [12] proposed the existence of a threshold level of MRF expression to initiate the fusion step, our results support the idea that this threshold level is reached earlier in the presence of Cr, allowing the myoblasts to differentiate more rapidly and attain swiftly the fusion step.

In contrast to MRF expression, IGF-I mRNA remained unchanged in control conditions while it rose about threefold after 72 h of incubation with Cr. Although it still remains unknown how Cr could activate the transcription of the IGF-I gene, the signalling pathways used by IGF-I to stimulate muscle growth has been recently characterised. The hypertrophic effect of IGF-I is mediated through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway [19], which leads to an increase in protein synthesis through the phosphorylation of p70S6k, a key regulator of S6 ribosomal subunit activity. Moreover, IGF-I also contributes to myogenesis by activating myogenin through the PI3K-Akt pathway [19]. On the other hand, a role of the mitogen-activated protein kinase [20] and the calcineurin pathways has also been suggested in the hypertrophic effect of IGF-I [21]. In the presence of Cr, the level of IGF-I mRNA was increased over the whole investigated period of time (72 h), whereas the mRNA levels of the various MRFs were only augmented at precise moments (Fig. 3), suggesting a general activation mechanism for IGF-I and a much more regulated mechanism for MRF transcription. All together, our observations raise the possibility that IGF-I might mediate the effects of Cr on growth and differentiation of myogenic cells in culture.

Interestingly, clenbuterol, which exerts powerful anabolic effects on skeletal muscle, also stimulates IGF-I expression [22] and Cr accumulation in myoblasts in culture [23]. To date, the mechanism by which clenbuterol increases IGF-I remains unknown, but could be mediated by the accumulation of Cr within the cell.

Although it is likely that the responses of C_2C_{12} cells differ strongly from those of human mature muscle fibres, the ability of Cr to stimulate MRFs has also been demonstrated in vivo. Recently, resistance training in humans was shown to increase the level of myogenin and MRF-4 mRNA in the vastus lateralis muscle, an increase potentiated by oral Cr supplementation [24]. Furthermore, Hespel and al. [25] examined the effect of oral Cr supplementation on MRF expression during human leg immobilisation and rehabilitation. Their results showed that myogenin protein expression was increased after rehabilitation in placebo but not under Cr supplementation, while MRF-4 protein expression was increased with Cr but not with placebo. So, the effects of Cr on MRFs are not only observed in myogenic cell culture, but are also present in vivo, even if results in humans are contradictory for myogenin, probably due to the experimental protocols (rehabilitation vs training, and delays for biopsies). Nevertheless, the direct linkage between results from cultures of myogenic cells and from adult humans should be taken with caution.

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