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Original article

Growth factor and cytokine interactions in myogenesis.

Part II. Expression of IGF binding proteins and protein kinases essential for myogenesis in mouse C2C12 myogenic cells exposed to TNF- α and IFN- γ

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Abstract

The aim of the study was to examine potential interactions among IGF-I and proinflammatory cytokines, TNF- α and IFN- γ , in the regulation of local IGF-I bioavailability and cellular proteins mediating myogenic signals. We investigated levels of IGFBP-4, -5, -6, protein kinase Czeta (PKC ζ), p38 and extracellular signal-regulated kinase (ERK1/2) in differentiating mouse C2C12 myoblasts. IGF-I significantly stimulated expression of IGFBP-5. TNF- α and IFN- γ attenuated the expression of IGFBP-4 and -6 under basal conditions and in the presence of IGF-I, and inhibited IGF-I-induced IGFBP-5 expression during 5-day myogenesis. TNF- α and IFN- γ markedly attenuated p38 expression in the presence of IGF-I on the 5th day of myogenesis. When combined with IGF-I the cytokines exerted opposite effects on the PKC ζ level, i.e. TNF- α caused an increase, whereas IFN- γ reduced the cellular content of this kinase. Exposition of C2C12 myoblasts to IGF-I or cytokines led to the stimulation of ERK1/2 phosphorylation; however, both TNF- α and IFN- γ exerted an inhibitory effect on the activation of ERK1/2 in myoblasts cultured in the presence of IGF-I. We concluded as follows: i) TNF- α and IFN- γ present in the extracellular environment of differentiating C2C12 myoblasts can alter the local bioavailability of IGF-I by inhibiting the expression of IGFBP-4, -5, and -6, ii) the decrease in p38 expression and ERK1/2 phosphorylation in C2C12 myoblasts exposed to cytokines can lead to disturbances in IGF-I-regulated myogenesis.

Key words: extracellular signal-regulated kinase, IGF-I, IGF binding proteins, proinflammatory cytokines, p38, protein kinase Czeta

Introduction

Insulin-like growth factor-I (IGF-I) plays a key role in regulating skeletal muscle growth and differentiation, and in maintaining homeostasis of the adult muscle tissue (Duan et al. 2010). IGF-I promotes muscle differentiation by inducing myogenin, MRF-4 and MEF-2C genes, which causes an increase in structural gene expression and formation of myotubes (Ok-sbjerg et al. 2004). The majority of IGF-I in the circulation is biologically inactive because it is bound to a family of IGFBPs (IGF binding proteins), which binds IGF-I with a greater affinity than that of its tyrosine kinase receptor (Firth and Baxter 2002). The major actions of IGFBPs are: i) to serve as transporters of IGFs in the plasma and control their distribution in the extravascular compartment, ii) to prolong the half-life of IGFs and regulate their metabolic clearance, iii) to modulate directly the interaction between IGFs and their receptors, and thus, indirectly, to control the bioavailability of IGFs in a target tissue. It has been suggested that IGFBPs function as a reservoir for IGF-I that could control the local delivery of growth factor (Paye and Forsten-Williams 2006). All six IGFBPs are expressed during embryonic development of skeletal muscle; however, it is suggested that the principal role in IGF-I activity regulation is played by IGFBP-3, -4, -5 and -6 (Yin et al. 2004).

Studies on myogenic growth and differentiation have revealed two main intracellular pathways by which the signals might be transmitted: the phosphatidylinositol-3-kinase (PI3K) cascade and the Ras/Raf/MEK/ERK1/ERK2 cascade (Mourkioti and Rosenthal 2005). Several Ser/Thr kinases including protein kinase B and p38 are involved in the myogenic differentiation program (De Alvaro et al. 2008). Very recently, the atypical protein kinase Czeta (PKC ζ) has been described as a mediator of myogenesis (de Thonel et al. 2010).

Recent research has demonstrated that several proinflammatory cytokines have the potential to interact with signalling pathways that regulate anabolic processes in skeletal muscle (reviewed in Adams 2010). Since IGF-I is a major anabolic factor for skeletal muscle (Nystrom et al. 2009), a local deficit of IGF-I resulting from changes in its bioavailability and/or modification of the signal transduction pathway may disturb regenerative processes in muscle tissue. The aim of the present study was therefore to examine the cellular levels of IGFBP-4, -5, -6 as main binding proteins controlling IGF-I bioavailability and playing a key role in myogenic differentiation. The levels of intracellular proteins essential for the mitogenic and myogenic effects of IGF-I i.e. PKC ζ , p38 and ERK1/2 in differentiated C2C12 myoblasts were also assessed.

Materials and Methods

Induction of myogenesis and assessment of protein synthesis

Mouse C2C12 myoblast culture that reached 90% confluence was subjected to a 5-day-differentiation (switch to differentiation medium – 2% (v/v) horse serum HS/DMEM) in the presence of the experimental factors: IGF-I (30 nmol/l), TNF- α (1 ng/ml) and IFN- γ (1 ng/ml), all supplied by Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). To determine the changes in protein synthesis, 4-hour-labeling with [3H]leucine (ICN Polfa Rzeszów, final activity 1 μ Ci/ml) was used. The results in d.p.m. were expressed as a % of the value obtained in control conditions on the first day of differentiation.

Immunoblotting

Aliquots of whole cell extracts corresponding to 50 μ g of total protein were subjected to SDS-PAGE. Membranes with electrotransferred proteins were saturated, probed with an appropriate primary antibody (Santa Cruz Biotechnology), and were then exposed to the HP-conjugated secondary antibody (Santa Cruz Biotechnology). Blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's directions. The bands were scanned and analyses of optical density were performed using a Kodak EDAS 290/Kodak 1D 3.5 system (Eastman Kodak Company, Rochester, NY, USA). The optical density of the band of each studied protein was presented in arbitrary units. This semi-quantitative method allowed the level of studied proteins between control and experimental treatments to be compared.

Statistics

The data were expressed as means \pm S.E. and were representative of three separate experiments performed in triplicate. Student's t-test was used for the comparison of two means (treatment effect). For each assay individual values were used in statistical analysis with $n = 9$ /treatment condition. Statistical significance was set at $P < 0.05$.

Results

In C2C12 myoblast cultures subjected to a 5-day-differentiation an increase in protein synthesis was observed, i.e. in control conditions the rate of

protein synthesis on the 5th day amounted to 332% of the value obtained on the first day. IGF-I significantly stimulated protein synthesis in C2C12 myogenic cells during the 5 days of observation (373.2%, $P < 0.05$ vs control value on the same day). Exposure of C2C12 myoblasts to TNF- α modified protein synthesis, which was manifested by a slight increase in radiolabelled leucine incorporation on the 5th day of observation (376.2%, $P < 0.05$) whereas IFN- γ had no effect (339.4%, not significant vs control). In the presence of TNF- α and IFN- γ combined with IGF-I the rate of protein synthesis was comparable to the value detected on the same day in cultures subjected to differentiation in the presence of IGF-I (371.9% and 358.0%, respectively). Such an observation allowed the cytotoxic effect of cytokines in the present study to be excluded.

The cellular level of IGFBP-4 did not change significantly during the 5 days of differentiation of control or IGF-I-treated C2C12 myoblasts (Fig. 1). TNF- α and IFN- γ attenuated the expression of IGFBP-4 during the 5 days of differentiation. In the presence of IGF-I IFN- γ had a stronger inhibitory effect on IGFBP-4 than TNF- α .

The expression of IGFBP-5 did not change significantly during the 5 days of differentiation either in control C2C12 myoblasts or in the presence of TNF- α

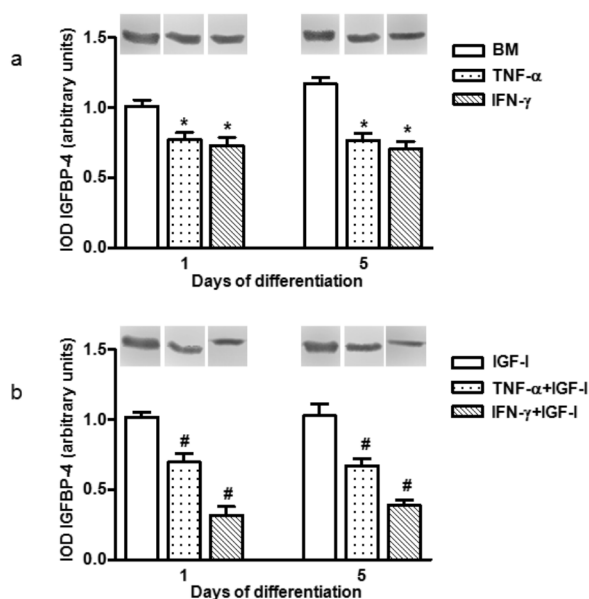


Fig. 1. Cellular content of IGFBP-4 in C2C12 myogenic cell cultures subjected to differentiation in 2% HS/DMEM (Basal medium, BM), supplemented with TNF- α (1 ng/ml) or IFN- γ (1 ng/ml) (a), and in the presence of IGF-I (30 nmol/l) (b). Blots are representative of three separate experiments. The densitometric quantitation of the specific bands is presented in arbitrary units, with the value obtained in the control group (Basal medium) set as 1. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

or IFN- γ (Fig. 2). IGF-I slightly but significantly stimulated expression of IGFBP-5 during the 5 days of myogenesis and both cytokines significantly inhibited IGF-I-induced IGFBP-5 expression during 5 days of myogenesis.

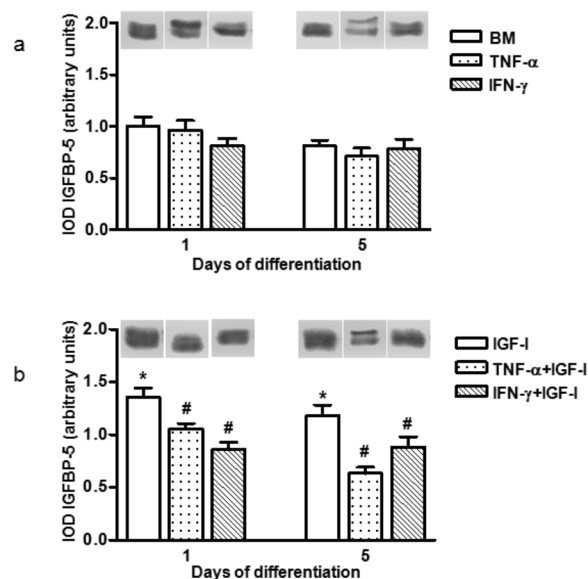


Fig. 2. Cellular content of IGFBP-5 in C2C12 myogenic cell cultures subjected to differentiation in the presence of 2% HS/DMEM (Basal medium, BM), supplemented with TNF- α (1 ng/ml) or IFN- γ (1 ng/ml) (a), and in the presence of IGF-I (30 nmol/l) (b). Blots are representative of three separate experiments. The densitometric quantitation of the specific bands is presented in arbitrary units, with the value obtained in the control group (Basal medium) set as 1. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

Exposure of myoblasts to TNF- α and IFN- γ significantly decreased IGFBP-6 level during the 5 days of myogenesis (Fig. 3). In cultures subjected to differentiation in the presence of cytokines and IGF-I a significant drop in IGFBP-6 level was noticed.

The cellular content of p38 increased during the 5-day myogenesis in IGF-I-treated cells (Fig. 4a). TNF- α and IFN- γ had no effect on p38 levels in basal conditions (not shown); however, both cytokines markedly attenuated p38 expression in the presence of IGF-I on the 5th day of myogenesis. Neither cytokine modified PKC ζ levels in basal conditions (not shown); however, when used in combination with IGF-I they exerted opposite effects. In C2C12 myoblasts exposed to TNF- α and IGF-I a significant increase in PKC ζ level was observed, whereas IFN- γ reduced the expression of this kinase (Fig. 4b).

TNF- α and IFN- γ did not evoke any significant effect on the ERK1/2 expression pattern in C2C12 myoblasts subjected to differentiation in the presence

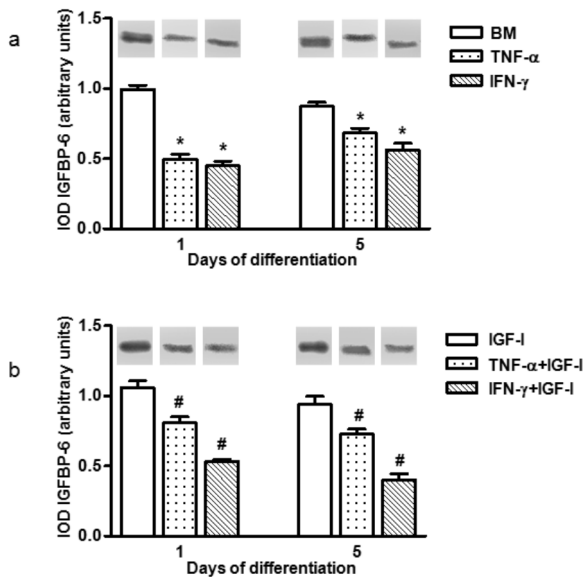


Fig. 3. Cellular content of IGFBP-6 in C2C12 myogenic cell cultures subjected to differentiation in the presence of 2% HS/DMEM (Basal medium, BM), supplemented with TNF- α (1 ng/ml) or IFN- γ (1 ng/ml) (a), and in the presence of IGF-I (30 nmol/l) (b). Blots are representative of three separate experiments. The densitometric quantitation of the specific bands is presented in arbitrary units, with the value obtained in the control group (Basal medium) set as 1. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

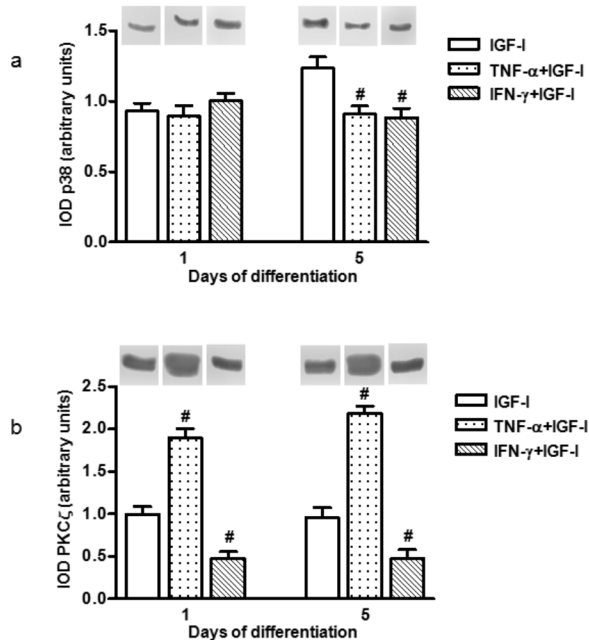


Fig. 4. Cellular content of protein kinase p38 (a) and PKC ζ (b) in C2C12 myogenic cell cultures subjected to differentiation in the presence of IGF-I (30 nmol/l), supplemented with TNF- α (1 ng/ml) or IFN- γ (1 ng/ml). Blots are representative of three separate experiments. The densitometric quantitation of the specific bands is presented in arbitrary units, with the value obtained in the control group (Basal medium) set as 1. # – significantly different *versus* IGF-I treatment on the same day.

of both 2% HS and IGF-I (Fig. 5). In control C2C12 myoblasts mostly the ERK2 (p42^{MAPK}) isoform was phosphorylated and this phosphorylation decreased during the 5 days of differentiation. Exposition of C2C12 myoblasts to IGF-I led to a significant stimulation of ERK1/2 phosphorylation. Treatment of C2C12 myoblasts with examined cytokines caused a phosphorylation of two ERK isoforms: ERK1 (p44^{MAPK}) and ERK2 (p42^{MAPK}). The slight but significant effect of IFN- γ on ERK1/2 phosphorylation was visible on the 1st day, whereas both TNF- α and IFN- γ caused a 2-fold increase in ERK1/2 phosphorylation on the 5th day of differentiation. In striking contrast, TNF- α and IFN- γ exerted an inhibitory effect on the activation of ERK1/2 in myoblasts cultured in the presence of IGF-I.

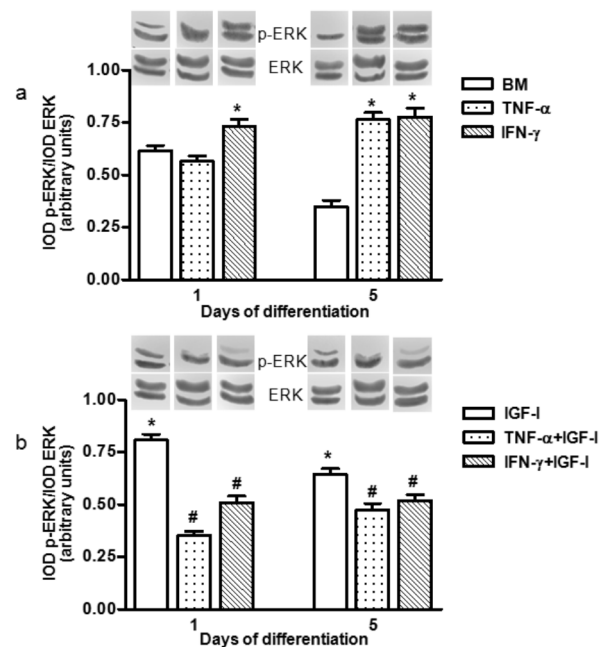


Fig. 5. Cellular content of the phosphorylated form of extracellular signal-regulated kinase 1/2 (p-ERK) in C2C12 myogenic cell cultures subjected to differentiation in the presence of 2% HS/DMEM (Basal medium, BM), supplemented with TNF- α (1 ng/ml) or IFN- γ (1 ng/ml) (a), and in the presence of IGF-I (30 nmol/l) (b). Blots are representative of three separate experiments. Total ERK 1/2 protein (ERK) at subsequent time points of the experiments is also presented. The densitometric quantitation of the phosphorylated ERK1/2 is presented in arbitrary units as a ratio: the density of the bands identified by the phosphospecific ERK1/2 antibody/the density of the bands identified by an antibody which recognizes total ERK1/2 protein. * – significantly different *versus* control value on the same day, # – significantly different *versus* IGF-I treatment on the same day.

Discussion

Although typically viewed in the context of immune modulation, cytokines are now known to be potent regulators of tissue metabolism, growth and differentiation, as well as repair and ageing (Jacobi et al.

2006, Solomon and Bouloux 2006). On the other hand, IGF-I appears to be critical in promoting both proliferation and differentiation of skeletal muscle cells, muscle hypertrophy and muscle regeneration following injury (Nystrom et al. 2009). In our recent study we found a reduction in differentiation of C2C12 myoblasts by TNF- α and IFN- γ , manifested by decreased MyoD levels and myoblast fusion, only in the presence of IGF-I (Wieteska-Skrzeczyńska et al., submitted). Such an observation indicates that in target cells the integration of numerous signals occurs by intracellular cross-talk among distinct ligand-activated systems and an activation of one pathway may interfere with the signal transduction of others, leading to the modulation or inhibition of the original biological effects.

In the current study addition of IGF-I to the differentiation medium resulted in a marked stimulation of the expression of IGFBP-5. A similar effect of IGF-I was observed previously during differentiation of several cell types (Tripathi et al. 2009), which indicates the regulatory role of IGF-I concerning the level of its binding proteins.

The catabolic response to excess of proinflammatory cytokines is frequently associated with an increase in IGFBP-1 (Thissen 2007) and a decrease in IGFBP-3 (Lang et al. 2002), whereas alterations in other IGFBPs, which play an important role in myogenesis regulation, remain inadequately explored. According to the present results, TNF- α and IFN- γ markedly reduced the expression of IGFBP-4 (Fig. 1), IGFBP-5 (Fig. 2) and IGFBP-6 (Fig. 3) in C2C12 myoblasts subjected to a 5-day-differentiation. The cytokines may be the primary stimulus for the reduction in expression of IGFBPs or the observed decrease in levels of IGFBPs may result from a reduction in the local IGF-I level in myoblasts treated with cytokines (Fernandez-Celemin et al. 2002) and/or from cytokine-induced resistance to IGF-I. It is noteworthy that TNF- α and IFN- γ decreased the expression of all examined IGFBPs, both of those which positively regulate IGF-I action in skeletal muscle (i.e. IGFBP-5) and those which attenuate IGF-I bioavailability at the level of skeletal muscle tissue (i.e. IGFBP-4 and -6). Thus, according to our results, it is difficult to point to one or another protein as being a *sine qua non* responsible for modification of the myogenic effect of IGF-I. It seems that in the local regulation of the IGF system the fine equilibrium between several BPs is much more important than exact levels of particular binding proteins, and that this could finally determine IGF-I bioavailability and activity in muscle cells. Jennische and Hall (2000) previously reported the increase of IGFBP-3, -4, -5, and -6 in regenerating rat skeletal muscle. Their results indicate that decreased expression of IGFBPs could potentially lead to the reduction in regenerative pro-

cesses, reflected by the inhibition of myogenesis found in our recent study (Wieteska-Skrzeczyńska et al. submitted). On the other hand, the inhibition of interactions of IGFBPs with systemic IGF-I has significant therapeutic potential for enhancing muscle repair after muscle injury (Schertzer et al. 2007). In this regard, the cytokine-evoked decrease in all examined IGFBPs observed in our study could be interpreted as a mechanism which was activated in order to potentiate the growth factor effect. Decreased levels of all studied BPs in cultures exposed to TNF- α or IFN- γ and IGF-I seem to be specific and did not result from increased cell mortality. Assessment of protein synthesis in all experimental treatments gave a similar pattern of results, which allow the cytotoxic effect to be excluded.

According to our recent study pretreatment of C2C12 myoblasts with proinflammatory cytokines impaired the acute metabolic effect of IGF-I and the activation of protein kinase B (Grzelkowska-Kowalczyk and Wieteska-Skrzeczyńska 2010). The aim of the present study was to examine the expression of other cellular proteins essential for the mitogenic, myogenic and metabolic effects of insulin and IGF-I in myoblasts under prolonged simultaneous treatment of TNF- α or IFN- γ and IGF-I.

The combined supplementation with cytokines and IGF-I caused a drop in p38 level which could lead to the reduction of myogenic differentiation under these conditions (Fig. 4a). On the other hand, the results presented in the current study did not support the involvement of PKC ζ in the differentiation of C2C12 myoblasts, since the effect of the studied cytokines on this kinase expression (stimulated by TNF- α and inhibited by IFN- γ , Fig. 4b) did not reflect alterations in myogenesis i.e. inhibition of MyoD, MHC and fusion index (Wieteska-Skrzeczyńska et al., submitted). This implicates the contribution of other signalling proteins leading to the inhibition of differentiation; nevertheless, PKC ζ has been described as regulating the cdk5/p35 signalling complex, an essential factor for myogenesis (de Thonel et al. 2010).

ERK may play dual role in myogenesis: it inhibits differentiation at the early stage but promotes myoblast fusion at the late stage of differentiation (Jo et al. 2009). In control cultures of C2C12 myoblasts the phosphorylation of ERK1/2 decreased gradually during the 5 days of differentiation (Fig. 5). Such an observation is consistent with the previous study of Adi and coworkers (2002) who demonstrated that in rat L6E9 skeletal myoblasts the ERK1/2 phosphorylation initially increases, but subsequently, as differentiation of myoblasts proceeds, the phosphorylation level of this kinase decreases. It should be noted, however, that terminal differentiation and fusion in the final stages of myogenesis require some degree of ERK1/2 phosphorylation to proceed (Tiffin et al. 2004). The

examined cytokines markedly increased ERK1/2 activation. Results concerning elevated phosphorylation of ERK1/2 in the presence of cytokines are compatible with the observation that sepsis or a constant intravenous infusion of TNF- α for 24 hours results in an increase in ERK1/2 phosphorylation in rat skeletal muscle (Vary et al. 2004). Moreover, in myotubes subjected to 24-hour incubation with TNF- α , this cytokine induced the phosphorylation of ERK1/2 (Plaisance et al. 2008). Similarly, in vascular smooth muscle cells, increased phosphorylation of ERK1/2 was shown to be dependent on elevated levels of IFN- γ (Igarashi et al. 2007).

Both TNF- α and IFN- γ exerted an inhibitory effect on IGF-I-stimulated phosphorylation of ERK1/2, that correspond to the reduction of IGF-I-dependent myogenesis evoked by these cytokines. Decreased ERK1/2 phosphorylation could result from impaired IGF-I action *per se* induced in the presence of cytokines or, alternatively, could be associated with the drop in IGF-BPs (Figs. 1-3). Such a hypothesis has previously been proposed by Ning and coworkers (2006) demonstrating that triple knockout mice lacking IGFBP-3, -4, and -5 displayed a tissue-specific decline in activation of the ERK signalling pathway.

Taken together our present results indicate subsequent mechanisms of the modulatory effect of proinflammatory cytokines on skeletal muscle growth and metabolism. The cytokine-induced modifications of acute hormone and growth factor effects (Hirosumi et al. 2004, O'Connor et al. 2008) as well as the inhibition of local IGF-I expression in skeletal muscle cells (Fernandez-Celemin et al. 2002) have already been described. Here, using the *in vitro* model of skeletal muscle, we supported the hypothesis that the expression of both IGF-BPs and key protein kinases could be targeted by proinflammatory cytokines present in the extracellular environment of differentiating myoblasts. Changes in the cellular levels of binding proteins and protein kinases could disturb IGF-I dependent myogenesis and could contribute to development of cytokine-mediated insulin and IGF-I resistance in mature myotubes.

We concluded as follows: i) TNF- α and IFN- γ present in the extracellular environment of differentiating C2C12 myoblasts can alter the local bioavailability of IGF-I by inhibiting the expression of IGFBP-4, -5, and -6, ii) the decrease in p38 expression and ERK1/2 phosphorylation in C2C12 myoblasts exposed to cytokines can lead to disturbances in IGF-I-regulated myogenesis.

Acknowledgements

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