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Research article

TREATMENT WITH TNF-α AND IFN-γ ALTERS THE ACTIVATION OF SER/THR PROTEIN KINASES AND THE METABOLIC RESPONSE TO IGF-I IN MOUSE C2C12 MYOGENIC CELLS

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Abstract: The aim of this study was to compare the effects of TNF-α, IL-1β and IFN-γ on the activation of protein kinase B (PKB), $p70^{S6k}$, mitogen-activated protein kinase (MAPK) and $p90^{rsk}$, and on IGF-I-stimulated glucose uptake and protein synthesis in mouse C2C12 myotubes. 100 nmol/l IGF-I stimulated glucose uptake in C2C12 myotubes by 198.1% and 10 ng/ml TNF-α abolished this effect. Glucose uptake in cells differentiated in the presence of 10 ng/ml IFN-γ increased by 167.2% but did not undergo significant further modification upon the addition of IGF-I. IGF-I increased the rate of protein synthesis by 249.8%. Neither TNF-α nor IFN-γ influenced basal protein synthesis, but both cytokines prevented the IGF-I effect. 10 ng/ml IL-1β did not modify either the basal or IGF-I-dependent glucose uptake and protein synthesis. With the exception of TNF-α causing an 18% decrease in the level of PKB protein, the cellular levels of PKB, $p70^{S6k}$, $p42^{MAPK}$, $p44^{MAPK}$ and $p90^{rsk}$ were not affected by the cytokines. IGF-I caused the phosphorylation of PKB (an approximate 8-fold increase above the basal value after 40 min of IGF-I treatment), $p42^{MAPK}$ (a 2.81-fold increase after 50 min), and the activation of $p70^{S6k}$ and $p90^{rsk}$, manifesting as gel mobility retardation. In cells differentiated in the presence of TNF-α or IFN-γ, this IGF-I-mediated PKB and $p70^{S6k}$ phosphorylation was significantly diminished, and the increase in $p42^{MAPK}$ and $p90^{rsk}$ phosphorylation

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Abbreviations used: IFN-γ – interferon-γ; IGF-I – insulin-like growth factor-I; IL-1β – interleukin-1β; MAPK – mitogen-activated protein kinase; PI-3K – phosphatidylinositol 3-kinase; PKB (Akt) – protein kinase B/Akt; TNF-α – tumour necrosis factor-α

was prevented. The basal $p42^{MAPK}$ phosphorylation in C2C12 cells treated with IFN-γ was high and comparable with the activation of this kinase by IGF-I. Pre-treatment of myogenic cells with IL-1β did not modify the IGF-I-stimulated phosphorylation of PKB, $p70^{S6k}$, $p42^{MAPK}$ and $p90^{rsk}$. In conclusion: i) TNF-α and IFN-γ, but not IL-1β, if present in the extracellular environment during C2C12 myoblast differentiation, prevent the stimulatory action of IGF-I on protein synthesis. ii) TNF-α- and IFN-γ-induced IGF-I resistance of protein synthesis could be associated with the decreased phosphorylation of PKB and $p70^{S6k}$. iii) The activation of glucose uptake in C2C12 myogenic cells treated with IFN-γ is PKB independent. iv) The similar effects of TNF-α and IFN-γ on the signalling and action of IGF-I on protein synthesis in myogenic cells could suggest the involvement of both of these cytokines in protein loss in skeletal muscle.

Key words: Cytokines, Glucose transport, IGF-I resistance, Protein synthesis, Signalling pathways

INTRODUCTION

Skeletal muscle wasting is observed in various pathological conditions, including infection, inflammation, type 1 diabetes, cancer and AIDS [1-3]. The potential mediators of this phenomenon are proinflammatory cytokines: TNF-α, IL-1β, IL-6 and IFN-γ [4, 5]. The concentration of TNF-α increases markedly in inflammatory conditions [6, 7] and cancer-associated cachexia [8]. A relationship between an increased level of TNF-α and the loss of muscle mass in the elderly has also been demonstrated [9]. Cytokines affecting the functions of myogenic cells may be produced by myocytes [10] or by other cells present in the muscle tissue, including neutrophils, macrophages, fibroblasts and endothelial cells [11]. The receptors specific for TNF-α, IL-1, IL-6 and IFN-γ are expressed in the skeletal muscle and myogenic cells at a low level, but their contents have been shown to increase noticeably after the induction of inflammation and treatment with a mixture of cytokines *in vitro* [12], suggesting the existence of local mechanisms enhancing the skeletal muscle response to cytokines.

The mechanisms of the catabolic effect of cytokines in the skeletal muscle may involve: a modulation of the activity of the hormones controlling protein metabolism [6]; the inhibition of the production of anabolic factors [13]; and the regulation of the expression of other cytokines [14]. In recent years, there has been a hypothesis regarding hormones and cytokines, which may mutually modulate biological effects via common intracellular signal transduction pathways. A simultaneous stimulation of the receptors specific for a given hormone and cytokine may abolish their biological effects. For example, TNF-α has been shown to inhibit the activation of components in the signal transduction emanating from the insulin receptor [15].

IGF-I is the major anabolic factor for the skeletal muscle [16]. Therefore, a drop in IGF-I level, any decrease in its bioavailability, and/or the inhibition of the intracellular transduction pathways activated by IGF-I may lead to the type of muscle wasting commonly observed in catabolic conditions. The biological activity of IGF-I requires its binding to a specific receptor that structurally resembles the insulin receptor [17]. One of the intracellular signalling pathways activated by the binding of IGF-I to this receptor is the Ras-ERK cascade via the Shc protein. This leads to an increase in the activity of the transcription factors and the stimulation of cell proliferation [18]. The second pathway induced by IGF-I is a cascade initiated through the phosphorylation of IRS proteins, leading to PI-3 kinase activation and the Ser^{473} phosphorylation of PKB, which mediates metabolic [19] and anti-apoptotic effects [20].

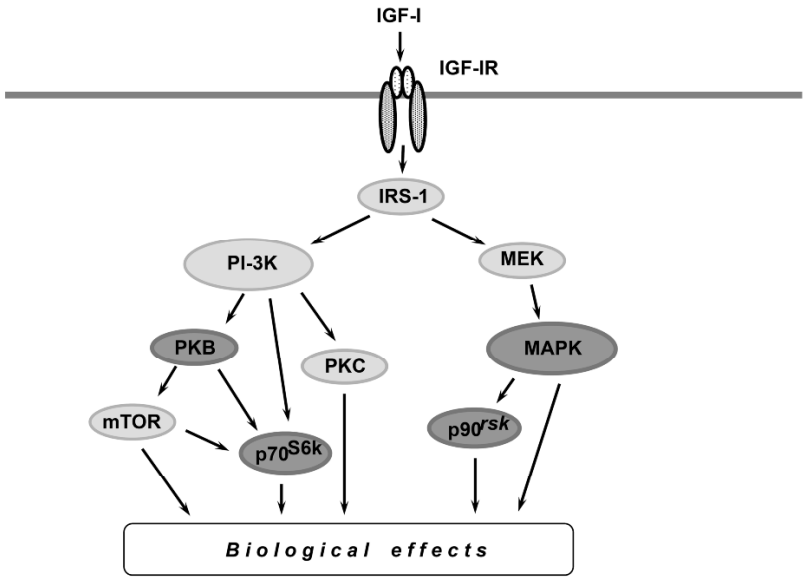

Fig. 1. A schematic representation of the post-receptor events in the IGF-I signalling system. The signalling elements analyzed in this study are marked in dark grey.

Keeping in mind that some actions of TNF-α are thought to be mediated by other cytokines [14], the aim of this study was to assess and to compare the effect of pre-treatment with TNF-α, IL-1β or IFN-γ on the activation of Ser/Thr protein kinases and on the metabolic response in mouse C2C12 myotubes stimulated with IGF-I. We investigated the basal and IGF-I-stimulated glucose uptake and protein synthesis, and the cellular content and phosphorylation of protein kinase B, $p70^{S6k}$, mitogen-activated protein kinase (MAPK) and $p90^{rsk}$ (Fig. 1).

MATERIALS AND METHODS

Chemicals

All the reagents were cell culture-tested and of high purity. The cell culture equipment was purchased from Corning-Costar Inc. (USA). The cell culture media, buffers, sera and antibiotics were obtained from Gibco Life Technologies. [1,2-^{3}H]2-deoxy-D-glucose (specific activity 60 Ci/mmol) and [4,5-^{3}H]L-Leucine (specific activity 42,5 Ci/mmol) were supplied by ICN Polfa Rzeszów. The antibodies were obtained from Santa Cruz Biotechnology. Sequi-Blot PVDF membrane 0.2 μm and all the reagents for immunoblotting were supplied by Bio-Rad Laboratories (CA, USA). TNF-α, IL-1β, IFN-γ, IGF-I (human recombinant) and other chemicals were purchased from Sigma Chemical Co.

Cell culture

The C2C12 mouse myoblast cell line (satellite cells from the thigh muscle), purchased from the European Collection of Animal Cell Cultures (ECACC), was used for the study. The cells were maintained in an exponential phase of growth in 10% (v/v) FCS/DMEM, together with an antibiotic-antimycotic mixture (50 IU/ml penicillin G sodium salt, 50 μg/ml streptomycin sulphate, 20 μg/ml gentamycin sulphate, 6 μg/ml tylosin and 1 μg/ml fungizone), in controlled humidified air supplemented with 5% CO_2, at 37ºC. The growth medium was changed every other day until the cells reached 100% confluence. The cells were then subjected to differentiation in a differentiation medium (2% (v/v) horse serum HS/DMEM) in the presence of 10 ng/ml TNF-α, IL-1β or IFN-γ for 5 days. The concentration of cytokines chosen for the study was based on the results of previous experiments demonstrating that such a dose did not affect cell viability, but (with exception of IL-1β) was effective in inhibiting the metabolic response to insulin [21 and unpublished data]. All the studied cytokines caused a transient stimulation of protein synthesis, meaning that their effects were limited to the first two days of treatment. In the cell cultures treated with TNF-α and IL-1β, the average fusion index assessed on the 3rd, 4th and 5th days of differentiation was similar to the control value, whereas IFN-γ decreased the fusion index on the 5th day by approximately 30% (data not shown). The control cultures were maintained in 2% HS/DMEM.

Glucose transport assay

Cells were seeded in 24-well plates at a concentration of 2 x 10^4/well. Differentiated myotubes, obtained as described above, were serum deprived for 3 h and then treated with 100 nmol/l IGF-I for 30 min. According to our previous observations, this concentration of IGF-I exerts a maximal stimulatory effect on protein synthesis in C2C12 myotubes [22]. The basal and IGF-I-stimulated glucose transport levels were determined via the incorporation of [1,2-^{3}H]2-deoxy-D-glucose (final activity 2 μCi/ml) during a 30-min incubation. The cells were washed five times with ice-cold PBS-D to stop the transport

assay. Then the cells were disrupted by the addition of 0.5 mol/l NaOH, mixed with scintillation liquid (Sigma Chemical Co.) and assessed for radioactivity in a β-counter (Packard Tri-CARB 1600 CA). The results obtained were corrected for non-carrier-mediated transport by measuring the glucose uptake in the presence of 0.01 mmol/l of cytochalasin B added just before the start of the transport assay.

Protein synthesis determination

The cells differentiated under the control conditions or in the presence of cytokines for 5 days were treated with 100 nmol/l IGF-I for 24 h. In order to determine the changes in protein synthesis, labeling with [^{3}H]leucine (at a final activity of 1 μCi/ml) was used for the last 4 h. The reaction was stopped by washing the cell cultures with ice-cold PBS-D, and then the cells were fixed with 5% TCA, washed twice with methanol, dissolved in 0.5 mol/l NaOH, and neutralized with 3 mol/l HCl. The resulting solutions were mixed with scintillation liquid and counted in a β-counter. The results in dpm were expressed as a percentage of the values obtained under the control conditions.

Immunoblotting

In order to assess the cellular content and activation of the protein kinases, the culture medium was removed on the 6th day of differentiation. The cells were maintained in basal medium (DMEM) for 3 h, and then treated with 100 nmol/l IGF-I for 10, 20, 30, 40 or 50 min. At the end of the treatment, the cells were washed with ice-cold PBS, scraped and centrifuged (1600 g, 6 min, 4ºC). The cell pellets were suspended in a lysis buffer (50 mmol/l Tris-acetate, 50 mmol/l NaF, 2.5 mmol/l EDTA, 1 mmol/l EGTA, 5 mmol/l sodium pyrophosphate, 5 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 2 mmol/l dithiothreitol, 1 mmol/l benzamidine, 4 μg/ml leupeptin, and 0.1% Triton X-100, pH 7.2). After a 30-min incubation at 4ºC, the lysates were centrifuged, and the protein content in the resulting supernatants was measured using the Bradford reaction (Bio-Rad Laboratories). For the immunoblot analysis, aliquots of the cell extracts corresponding to 50 μg of total protein were subjected to SDS-PAGE under reducing conditions. The electrotransfer of the separated proteins to the PVDF membrane was conducted for 1 h at 100 V. The membranes were then saturated in TBS buffer (20 mmol/l Tris-base, 500 mmol/l NaCl, pH 7.5) supplemented with 5% non-fat powdered milk for 1 h, and subsequently probed with an appropriate primary antibody for 16 h at 4ºC. After extensive washing in TBS containing 0.05% Tween-20, the membranes were exposed to the appropriate secondary antibody conjugated with horseradish peroxidase. The blots were developed using the Enhanced Chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's instructions. The bands were scanned, and analyses of the optical density were performed using Kodak ID Image Analysis Software (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 us to compare the level of total and/or phosphorylated protein kinases between the control and experimental treatments.

Statistical analysis

The data was expressed as a percentage of the changes related to the control or initial values ± SEM. The results were statistically evaluated using ANOVA and the multiple range Tukey test. The statistical significance was set as $P < 0.05$.

RESULTS

One hundred nmol/l IGF-I markedly stimulated glucose uptake in mouse C2C12 myotubes (198.1% of the basal value in the control group, $p < 0.05$, Fig. 2A). Pre-treatment with 10 ng/ml TNF-α did not significantly modify basal glucose uptake (85.5% of the control value), but it completely abolished the stimulatory effect of IGF-I (90.3% of the control value). Neither the basal nor IGF-I-stimulated glucose uptake levels were affected by pre-treatment with 10 ng/ml IL-1β (108.0% and 183.0% of the control value, respectively). Adding 10 ng/ml IFN-γ to the differentiation medium markedly activated glucose transport as assessed under basal conditions (167.2% of the control value, $p < 0.05$). The glucose uptake in cells differentiated in the presence of IFN-γ was not significantly further modified by the addition of IGF-I (185.0% of the control value).

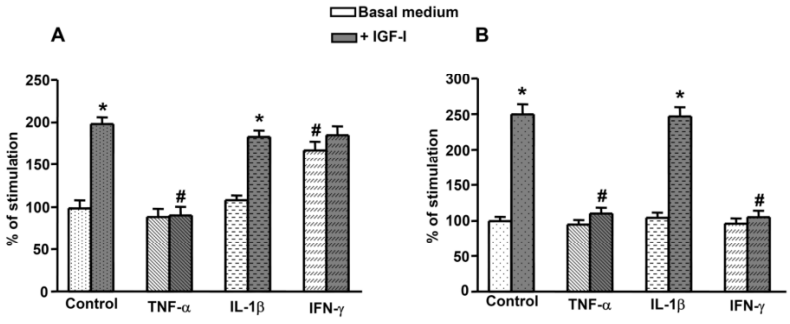

Fig. 2. The effect of 100 nmol/l IGF-I on glucose uptake (A) and protein synthesis (B) in mouse C2C12 myogenic cells pre-treated with TNF-α, IL-1β or IFN-γ. Mouse C2C12 myoblasts were subjected to a 6-day differentiation in the presence of the cytokines (each at a concentration of 10 ng/ml), as described in the Materials and Methods section. * – significantly different vs the corresponding treatment without IGF-I. # – significantly different vs the corresponding treatment without cytokines.

Supplementing the culture medium with IGF-I resulted in a significant increase in the rate of protein synthesis (249.8 % of the control value, $p < 0.05$, Fig. 2B). Pre-treating the cells with TNF-α did not influence the basal protein synthesis (95.2% of the control value) and completely prevented IGF-I stimulation of protein synthesis in C2C12 myotubes (110.3% of the control value). Similar

results were obtained with IFN-γ: it did not affect the basal rate of protein synthesis (96.3% of the control value) and it abolished the IGF-I-mediated activation of this process (105.5% of the control value). The addition of IL-1β to the culture medium did not modify either the basal or IGF-I-dependent protein synthesis (104.4% and 246.8% of the control value, respectively).

In view of the marked influence of some cytokines on the metabolic response to IGF-I, the next stage of the study aimed to investigate and to compare their effects on the cellular level and the activation of IGF-I signalling proteins. According to these current (Fig. 3 *Inset*) and previous [5] observations, the experimental factors used in this study had no effect on the cellular content of actin. Therefore, the alterations in the optical density of the bands result from the

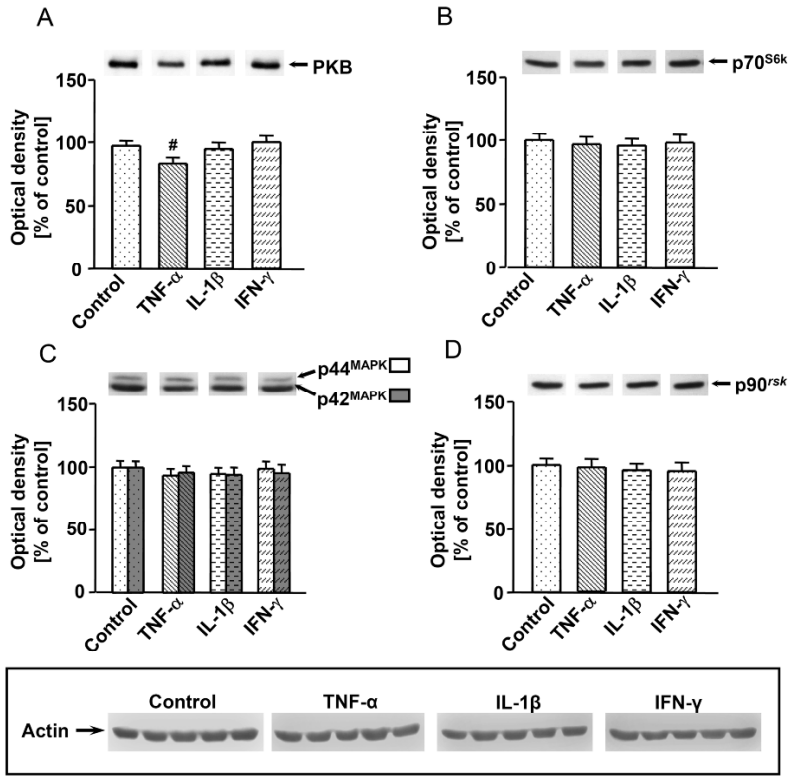

Fig. 3. The cellular content of the PKB (A), $p70^{S6k}$ (B), MAPK (C) and $p90^{rsk}$ (D) and actin during the 5 days of the experiment (*Inset)* in C2C12 myogenic cells subjected to differentiation in a basal medium (Control) or in the presence of TNF-α, IL-1β or IFN-γ (each at a concentration of 10 ng/ml). Immunoblotting analyses were performed using the appropriate polyclonal antibody. The blots are representative of three separate experiments. The densitometric quantitation of each of the proteins is presented in arbitrary units, with the value in the control group set as 100%. # – significantly different vs the control group.

specific modifications of the cellular level of the studied protein associated with the effect of the experimental factors. Pre-treatment of C2C12 myogenic cells with TNF-α caused a significant decrease in the PKB protein content (by 18% compared to the untreated culture, $p < 0.05$, Fig. 3). The cellular levels of the $p70^{S6k}$, $p42^{MAPK}$, $p44^{MAPK}$ and $p90^{rsk}$ proteins were not affected by TNF-α or by the other cytokines studied.

IGF-I caused the activation of protein kinase B as assessed by an immunoblot analysis using anti-phospho-PKB antibody. In the control myotubes, the highest

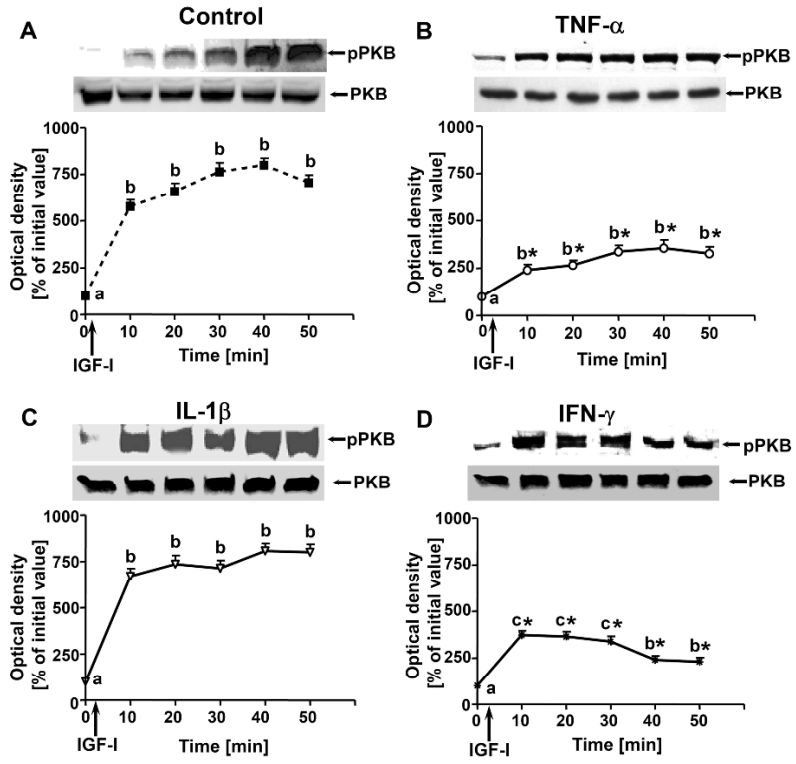

Fig. 4. IGF-I-stimulated PKB phosphorylation (pPKB) in C2C12 myogenic cells subjected to differentiation under control conditions (A), or in the presence of 10 ng/ml TNF-α (B), IL-1β (C) or IFN-γ (D). The immunoblotting analyses were performed using the anti-phospho-PKB (Ser^{473}) antibody. Total PKB protein (PKB) at subsequent time points of the experiments is also presented. The blots are representative of three separate experiments. The densitometric quantitation of the phosphorylated PKB is presented in arbitrary units as a ratio: the density of the bands identified by the phosphospecific PKB antibody/the density of the bands identified by an antibody which recognizes total PKB protein. The value obtained at time 0 for each group was set as 100%. The results are expressed as the means ± SEM of three separate experiments. a, b, c – values described with different letters for the same cell treatment differ significantly ($p < 0.05$). * – significantly different vs the control group at the same time point.

phosphorylation/activation of PKB occurred after 40 min of IGF-I treatment, when an approximately 8-fold increase above a value obtained at time 0 was noted (Fig. 4A). In cells differentiated in the presence of TNF-α or IFN-γ, the IGF-I-mediated PKB phosphorylation was significantly diminished, i.e. in the whole observation period, the phosphorylation level of PKB was approximately half that in the control culture (Fig. 4B, D).

When extracted from untreated cells and subjected to SDS-PAGE, the $p70^{S6k}$ appeared as a single band with an apparent molecular mass of 70 kDa. Treating the C2C12 myotubes with IGF-I also led to the activation of $p70^{S6k}$, the kinase lying downstream of PKB (Fig. 5A). The activation of $p70^{S6k}$ caused by multiple phosphorylation results in several phosphorylated forms that exhibit decreased electrophoretic mobility. Exposing the C2C12 control cells to IGF-I resulted in a gel mobility retardation of $p70^{S6k}$, indicating its phosphorylation and activation. In cells differentiated in the presence of TNF-α or IFN-γ, a 2-fold decrease in IGF-I-mediated $p70^{S6k}$ phosphorylation was observed (Fig. 5B, D).

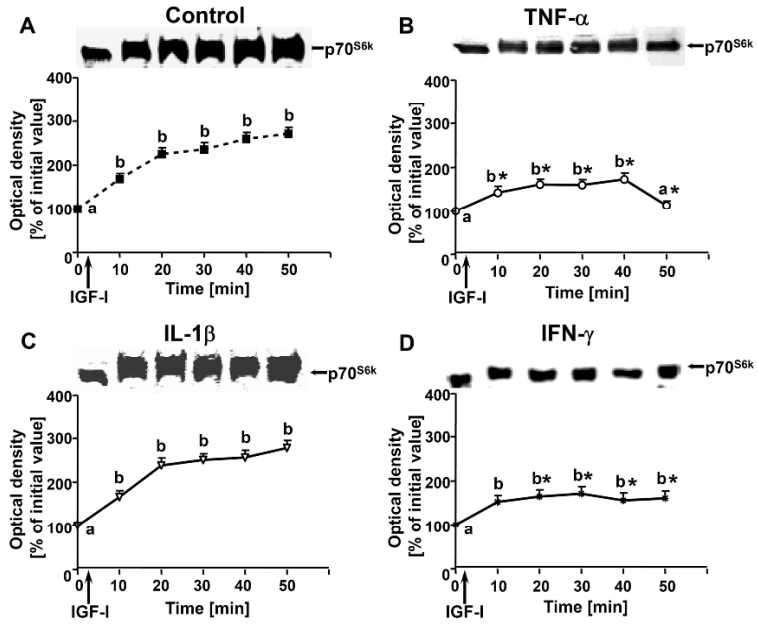

Fig. 5. IGF-I-stimulated $p70^{S6k}$ phosphorylation in C2C12 myogenic cells subjected to differentiation under control conditions (A), or in the presence of TNF-α (B), IL-1β (C) or IFN-γ (D). The immunoblotting analyses were performed using polyclonal anti-$p70^{S6k}$ antibody. The blots are representative of three separate experiments. The densitometric quantitation of $p70^{S6k}$ is presented in arbitrary units with the value obtained at time 0 for each group set as 100%. The results are expressed as the means ± SEM of three separate experiments. a, b – values described with different letters for the same cell treatment differ significantly ($p < 0.05$). * – significantly different vs the control group at the same time point.

In the control C2C12 myogenic cells treated with IGF-I, a marked increase in the phosphorylation of the p42 isoform of MAP kinase was noted (Fig. 6A). The highest IGF-I-dependent phosphorylation of $p42^{MAPK}$ was 2.81-fold higher than the control level, and was observed after 50 min of treatment. A 6-day differentiation of myogenic cells in the presence of TNF-α prevented this increase in $p42^{MAPK}$ phosphorylation (Fig. 6B). The basal $p42^{MAPK}$ phosphorylation in the C2C12 cells treated with IFN-γ was high and comparable with the phosphorylation of this kinase in the control culture after IGF-I stimulation (Fig. 6D).

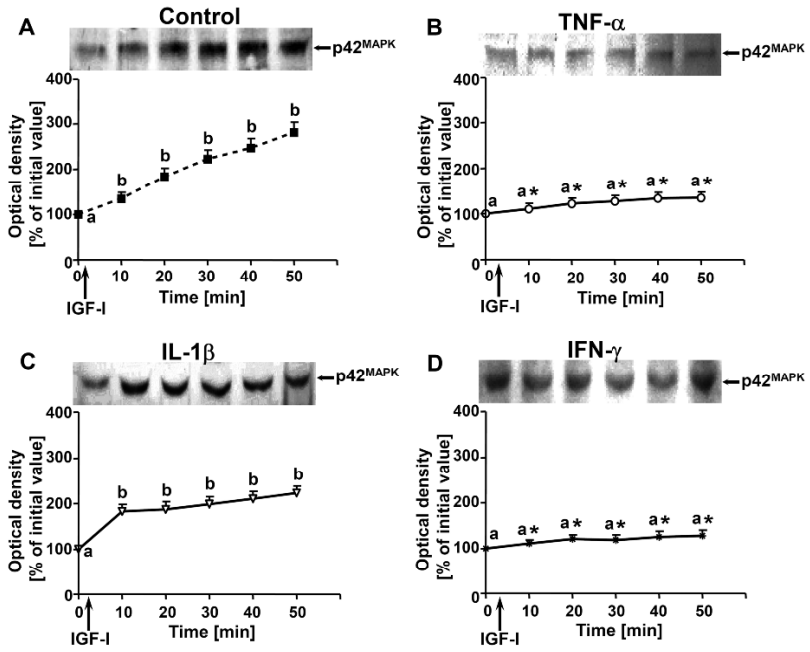

Fig. 6. IGF-I-stimulated MAPK phosphorylation in C2C12 myogenic cells subjected to differentiation under control conditions (A), or in the presence of TNF-α (B), IL-1β (C) or IFN-γ (D). The immunoblotting analyses were performed using the anti-phospho-ERK (MAPK) antibody. The blots are representative of three separate experiments. The densitometric quantitation of the phosphorylated $p42^{MAPK}$ is presented in arbitrary units as a ratio: the density of the bands identified by the phosphospecific MAPK antibody/the density of the bands identified by an antibody which recognizes total MAPK protein. The value obtained at time 0 for each group was set as 100%. The results are expressed as the means ± SEM of three separate experiments. a, b – values described with different letters for the same cell treatment differ significantly ($p < 0.05$). * – significantly different vs the control group at the same time point.

Adding IGF-I to the differentiation medium also led to the activation of $p90^{rsk}$, a signalling element lying downstream of MAP kinase, which was manifested by gel mobility retardation (Fig. 7A). However, no activation of $p90^{rsk}$ was

observed in cells preincubated with TNF-α or IFN-γ (Fig. 7B, D). As expected, pre-treatment of myogenic cells with IL-1β did not modify the IGF-I-stimulated phosphorylation of PKB (Fig. 4C), $p70^{S6k}$ (Fig. 5C), $p42^{MAPK}$ (Fig. 6C) or $p90^{rsk}$ (Fig. 7C).

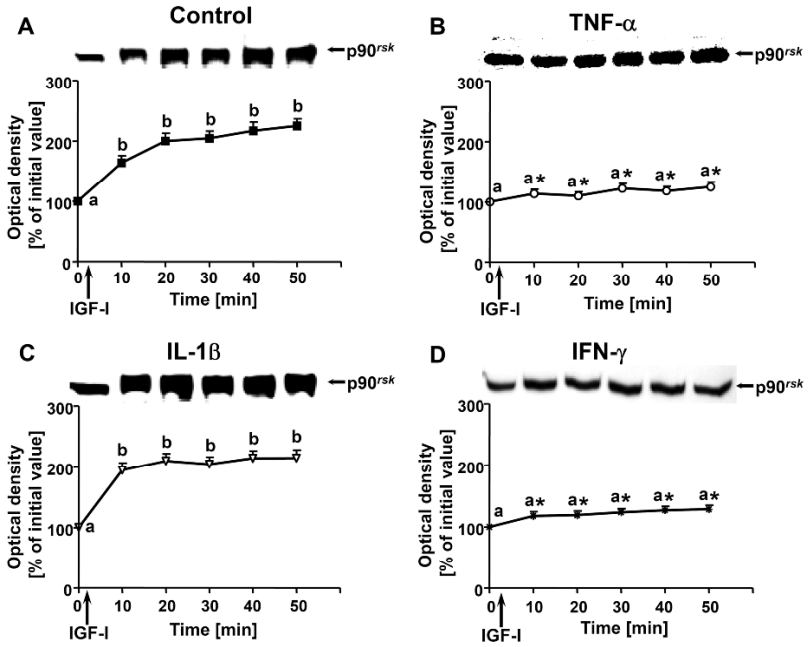

Fig. 7. IGF-I-stimulated $p90^{rsk}$ phosphorylation in C2C12 myogenic cells subjected to differentiation under control conditions (A), or in the presence of TNF-α (B), IL-1β (C) or IFN-γ (D). The immunoblotting analyses were performed using polyclonal anti-$p90^{rsk}$ antibody. The blots are representative of three separate experiments. The densitometric quantitation of $p90^{rsk}$ is presented in arbitrary units with the value obtained at time 0 for each group set as 100%. The results are expressed as the means ± SEM of three separate experiments. a, b – values described with different letters for the same cell treatment differ significantly ($p < 0.05$). * – significantly different vs the control group at the same time point.

DISCUSSION

Cachexia is attributed in part to an elevation in the level of circulating proinflammatory cytokines. TNF-α is the prototype in this category, but other cytokines are also thought to play a role [14]. The effects of cytokines on skeletal muscle catabolism can involve the modification of the anabolic action of hormones and growth factors.

The results of most of the experiments on the effects of TNF-α revealed reduced muscle protein synthesis, increased muscle protein breakdown or a combination of the two, leading to a net skeletal muscle loss; however, a direct effect of TNF-α

on protein turnover in muscle is still controversial. Pre-treatment of septic rats with the TNF-α antagonist TNF-binding protein did not prevent increased expression of two muscle-specific E3 ligases, MuRF1 and atrogin-1, leading to the conclusion that under *in vivo* conditions in mature adult rats, the sepsis-induced increase in these atrogenes is independent of TNF-α [23]. In mouse myotubes after acute TNF-α treatment [24] and in the cardiac muscle of septic rats [25], the initiation of protein translation was profoundly impaired. However, TNF-α has more recently been found to cause an increase in the protein content and fractional synthesis rate by enhancing protein translation in mouse myotubes *in vitro* [26]. An increased total protein content with a reduced fast myosin accumulation in myotubes treated with TNF-α was also claimed [27]. In a recent study, a 4-h infusion of recombinant human TNF-α did not affect protein synthesis, breakdown and net degadation either in the whole body or in the skeletal muscle in humans [28]. In this study, however, only the acute effect of TNF-α and the moderate elevation in its plasma level, far less than that seen in sepsis and chronic diseases, was evaluated. It cannot be excluded that the chronic role of TNF-α on muscle catabolism is different and/or mediated by unknown factors. Interestingly, chronic inflammation altered the expression patterns of genes involved in protein turnover in human skeletal muscle: genes contributing to proteasome functions were slightly up-regulated, and genes involved in protein synthesis were mildly down-regulated [29].

Our recent observations demonstrated that high concentrations of TNF-α inhibit insulin-regulated glucose uptake and protein synthesis in C2C12 myotubes [21]. In this study, we found evidence supporting the idea that this is also the case in the metabolic response of myotubes to IGF-I (Fig. 2). Noteably, in our study, 10 ng/ml TNF-α did not modify the basal rate of either glucose uptake or protein synthesis, which allows us to exclude the possibility that the cytokine has a cytotoxic action at this concentration. Consistent with this observation, TNF-α alone at concentrations ranging between 0.1 and 10 ng/ml was not cytotoxic for C2C12 myoblasts, as assessed by the propidium iodide and Hoechst 33342 staining methods [30].

The lack of alterations in the basal rate of protein synthesis in the presence of TNF-α is in striking contrast to recent observations which revealed enhanced protein translation in myotubes treated with this cytokine [26]. The discrepancy between the results on the effect of TNF-α on muscle protein turnover has a few possible explanations, but usually results from the cell model studied, the culture method used, and the differentiation state of the cells. In both studies, the effect of TNF-α was examined in C2C12 myotubes, but we assessed the protein synthesis on the 5^{th} day of differentiation after 5 days of treatment, whereas Plaisance *et al.* [26] found TNF-α-mediated stimulation of this process in myotubes on the 8^{th} day of differentiation after 48 h of cytokine treatment. A difference between the short- and long-term effect could be at least partly explained by the changes in receptor function and/or ligand binding, with

transient increasing expression of TNF-R1 leading to enhanced protein synthesis, followed by induction of feedback loops, such as the secretion of soluble TNF-R, which sequesters the cytokine [26]. Alternatively, a decrease in autocrine IGF-I production [13, 42] could blunt the increase in protein synthesis.

The TNF-α concentration effective in causing a decrease in IGF-I-stimulated glucose uptake and protein synthesis can be considered as physiological, i.e. in the range of those found in inflammatory states [31, 32]. Besides, when possible paracrine interactions between the cytokine-producing cells and myocytes are considered, local concentrations could be even higher than those found in circulation in inflammatory states.

TNF-α is not the only cytokine capable of inducing a catabolic state, and some of its actions have been shown to be mediated by other cytokines [14]. Therefore, we studied the effect of IL-1β and IFN-γ on IGF-I signalling and metabolic effects in C2C12 myogenic cells. In the experiments presented here, IL-1β failed to modify the glucose uptake and protein synthesis stimulated by IGF-I (Fig. 2), and these results resemble the lack of effect of this cytokine on insulin action observed in our previous study [21]. IFN-γ reduced IGF-I-stimulated protein synthesis in C2C12 myogenic cells. In fact, an inhibitory action of IFN-γ on muscle protein translation had recently been described [33], and those authors had indicated this mechanism as a molecular explanation for the reduced muscle growth during infection. In that study, however, IFN-γ was used in combination with lipopolysaccharide, whereas in our study, we established the direct effect of IFN-γ in the reduction of IGF-I-stimulated protein synthesis in cultures of C2C12 myogenic cells.

Evidence has already been accumulated that IFN-γ may act as a modulator of TNF-α signalling and effects. TNF-α and IFN-γ reduce myosin expression in myotubes and murine skeletal muscle [5]. Recent data indicated that TNF-α alone or in combination with IFN-γ induced apoptosis in skeletal muscle and in differentiating myocytes [34]. In another study, IFN-γ was found to inhibit the TNF-α-induced apoptosis of murine myotubes, which makes this cytokine a potential tool in the therapy of muscle atrophy [35]. Recently described marked differences in myotube responses to TNF-α and IFN-γ concerning protein kinase activities, the myosin heavy chain content, and the total protein-to-DNA ratio prompted previous researchers to exclude the direct catabolic effect of IFN-γ on the muscle [36]. In contrast to this latter idea, in our current study, we found a marked similarity in the modifications of key intracellular IGF-I signalling pathways and protein synthesis in myogenic cells differentiating in the presence of TNF-α or IFN-γ.

To investigate the signalling steps activated by IGF-I, which in turn, could be potentially altered by pre-treatment with TNF-α or IFN-γ, we considered protein kinase B, since this kinase propagates insulin/IGF-I signalling by phosphorylating downstream effectors and by phosphorylating IRS, thus generating a positive-feedback loop [37]. In this study, IGF-I caused the

activation of PKB in control myotubes, and this effect was significantly diminished in cells differentiated in the presence of TNF-α or IFN-γ (Fig. 4). Such an observation suggested that PKB may play an essential role in IGF-I resistance resulting from pre-treatment with these cytokines. Similar results have been shown in primary rat myotubes, where, upon insulin stimulation, PKB was highly serine and threonine phosphorylated, and both effects were impaired by TNF-α pre-treatment [38]. The decreased PKB phosphorylation after a 6-h treatment of muscle cells with IFN-γ has also been described [36]. According to observations performed on adipocytes, PKB-dependent insulin signalling may be impaired upon TNF-α exposure due to increased ubiquitination of PKB protein leading to its degradation through the 26S proteasome [41], which is compatible with the decrease in the PKB protein level in C2C12 cells treated with TNF-α in our current study (Fig. 3A). Interestingly, the effect of TNF-α on signalling proteins seems to be time-dependent, since neither the PKB protein content nor PKB phosphorylation were altered in L6 myotubes and VSMC exposed to high concentrations of this cytokine for 6 h [40].

In this study IGF-I activated $p70^{S6k}$, which manifested in the appearance of several phosphorylated forms that exhibit decreased electrophoretic mobility (Fig. 5). In cells differentiated in the presence of TNF-α or IFN-γ, an approximately 2-fold decrease in IGF-I-mediated $p70^{S6k}$ phosphorylation was noted, suggesting the role of $p70^{S6k}$ in the regulation of the metabolic response to IGF-I in C2C12 myogenic cells. This impaired $p70^{S6k}$ activation could mirror the activity of upstream IGF-I signal transduction components, i.e. PKB, one of the important signalling inputs implicated in the activation of $p70^{S6k}$ [41]. Moreover, in agreement with our observations, it was recently postulated that the inhibition of protein synthesis by a combination of IFN-γ and LPS can occur via the down-regulation of signalling from the mammalian target of rapamycin (mTOR) [33], an element lying upstream of $p70^{S6k}$. Thus, the inflammatory stimuli appear to alter the ability of IGF-I signals to be efficiently transduced at the cellular level. These changes may in part be responsible for the reduction in muscle protein synthesis during infection and other conditions associated with the overproduction of cytokines. This is consistent with the results from mouse myotubes treated with TNF-α [24] and from the cardiac muscle of septic rats [25], where, in the presence of high concentrations of the cytokine, $p70^{s6k}$ phosphorylation and eIF4E activity, the key factors for the initiation of translation, were markedly reduced. It should also be noted that the recently reported enhancement of protein synthesis in C2C12 myotubes treated with TNF-α [26] was accompanied by the activation of $p70^{S6k}$.

We also looked at potential modifications in the MAPK/$p90^{rsk}$ pathway in C2C12 myogenic cells exposed to cytokines. The increase in $p42^{MAPK}$ phosphorylation seen after stimulation with IGF-I did not appear in cells differentiating in the presence of TNF-α or IFN-γ (Fig. 6). The basal $p42^{MAPK}$ phosphorylation in C2C12 cells treated with TNF-α was constant during the

observation period, and in the case of IFN-γ, was comparable with the phosphorylation of this kinase in the control culture after IGF-I stimulation. These results are partly consistent with those from previous studies demonstrating that short-term exposure to TNF-α had no effect on the MAPK protein content and phosphorylation in C2C12 myotubes [42] and in rat L6 myotubes [40]. The effect of IFN-γ on $p42^{MAPK}$ phosphorylation seems to be time-dependent: in another study, this cytokine at 60 ng/ml did not activate several isoforms of MAPK (i.e. JNK, ERK1/2, p38) after a 6-hour exposure [36]. In our study, both TNF-α and IFN-γ prevented the activation of $p90^{rsk}$ (Fig. 7), and this effect was probably independent of MAPK.

The presence of IFN-γ during the process of differentiation of the myoblasts abolished their response to acute IGF-I stimulation with regard to protein synthesis (Fig. 2). Under the same conditions, there was a stimulation of glucose uptake by IFN-γ. The activation of glucose transport is interesting given the simultaneous impairment of protein synthesis and the phosphorylation of signalling elements essential for the IGF-I metabolic response. Such an observation indicates the selective action of this cytokine on specific signalling pathways and metabolic processes rather than general inhibition due to a cytotoxic effect. Moreover, it is evident that alternative cellular pathways are responsible for regulating the glucose uptake during IFN-γ treatment. According to early observations, glucose uptake in L6 myotubes treated with IFN-γ was redox sensitive [43].

In view of the marked influence of TNF-α and IFN-γ on IGF-I signalling and metabolic response, the question arises whether the observed effects could be due to the alterations of myogenesis? According to our recent study, the differentiation of myoblasts, as defined by the expression of MyoD and myogenin, which are myogenic transcription factors critical for the initiation of myoblast differentiation, as well as by the fusion index, was impaired in TNF-α- and IFN-γ-treated cells [44]. Thus, the TNF-α- and IFN-γ-exerted downregulation of IGF-I-activated signalling elements and the decrease in IGF-I-controlled storage of protein could at least partly result from the modification of myogenesis, which in turn, could alter the cellular pathways required for the response of differentiated myotubes to IGF-I. However, it should be noted that changes in the differentiation process are not sufficient to explain the IGF-I resistance of myotubes, since IL-1β, which was effective in inhibiting myogenesis [44], did not impair the metabolic response of C2C12 myotubes to IGF-I in our study.

Inflammatory stimuli appear to alter the ability of IGF-I signals to be efficiently transduced at the intracellular level. We concluded that: i) TNF-α and IFN-γ, but not IL-1β, if present in the extracellular environment during C2C12 myoblast differentiation, prevent the stimulatory action of IGF-I on protein synthesis. ii) The TNF-α- and IFN-γ-induced IGF-I resistance of protein synthesis could be associated with decreased phosphorylation of PKB and $p70^{S6k}$. iii) The IFN-γ-

mediated activation of glucose uptake in C2C12 myogenic cells is PKB independent. iv) The similar effects of TNF-α and IFN-γ on IGF-I signalling and protein synthesis in myogenic cells could suggest the involvement of both of these cytokines in protein loss in skeletal muscle.

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