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Inhibitory Effects of IL-6 on IGF-1 Activity in Skeletal Myoblasts Could be Mediated by the Activation of SOCS-3

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ABSTRACT

In elderly people, low and high levels of insulin-like growth factor 1 (IGF-1) and interleukin-6 (IL-6), respectively, are well documented and may contribute to reduced muscle mass and poor muscle function of ageing and suggesting a biological interactions between IGF-1 and IL-6. However, the dual effect of IGF-1/IL-6 on skeletal muscle differentiation and proliferation has not been fully investigated. We therefore hypothesised that IL-6 impairs the biological activity of IGF-1 in skeletal muscle through inhibiting its signalling pathways, ERK1/2 and Akt. Our aim was to examine the combined effects of these factors on models of muscle wasting, with objectives to examine skeletal muscle differentiation and proliferation using the murine C2 skeletal muscle cell line. Cells were cultured with DM, IGF-1 and IL-6 alone (control treatments), or co-cultured with IGF-1/IL-6. Co-incubation of C2 cells in IGF-1 plus IL-6 resulted in maximal cell death ($22 \pm 4\%$; $P < 0.005$) compared with control treatments ($14 \pm 2.9\%$). This was also confirmed by cyclin D1 expression levels in co-incubation treatments ($7 \pm 3.5\%$; $P < 0.05$) compared with control treatments ($\sim 23\%$). The expression levels of myogenic-specific transcriptional factor mRNAs (myoD and myogenin) were also significantly ($P < 0.005$) reduced by 70% and 90%, respectively, under the co-incubation regimes, compared with control treatments. Signalling investigations showed significant phosphorylation reduction by 20%, ($P < 0.05$) of ERK1/2 and Akt in co-incubation treatments relative to either treatment alone. Expression studies for SOCS-3 (1.6-fold $\pm$ 0.08, $P < 0.05$) and IRS-1 (0.65-fold $\pm$ 0.13 $P < 0.005$) mRNAs showed significant elevation and reduction for both genes, respectively, in co-treatments relative to control treatments. These data may suggest that IL-6 exerts its inhibitory effects on IGF-1 signalling pathways (ERK1/2 and Akt) through blocking its receptor substrate IRS-1 by SOCS-3. J. Cell. Biochem. 113: 923–933, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: IL-6; IGF-1; MYOBLASTS; DIFFERENTIATION; CYCLIN D1

INSULIN-LIKE GROWTH FACTOR 1 (IGF-1)

Skeletal muscle displays a high degree of adaptation to the use (e.g. physical activity which induces IGF-1 signalling pathway) or disuse (e.g. aging which promotes IL-6 signalling pathways) [Cappola et al., 2003]. IGF-1 is produced both by the liver as an endocrine hormone and target tissues (e.g. skeletal muscle) where it acts locally in a paracrine/autocrine fashion [Hoppener et al., 1985]. Indeed, IGF-1 is a unique growth factor in that it initiates both proliferation and differentiation through the phosphorylation of its key receptor, insulin receptor substrate-1 (IRS-1). Proliferation is initiated not only during development, but throughout life as well as in cell culture models [Florini et al., 1996; Borst and Lowenthal, 1997; Musaro et al., 2001]. Whereas differentiation is activated during muscle-specific transcription factors are up-regulated, which culminate in the fusion of myoblasts into myotubes [Florini et al., 1996; Stewart et al., 1996; Coolican et al., 1997; Singh et al., 1999; Yakar et al., 2001]. Both of these key processes are reported to involve the phosphorylation and activation of extracellular signal-regulated Kinases 1/2 (ERK1/2), PI3K/Akt [Coolican et al., 1997; Foulstone et al., 2004] and their downstream targets which have previously been implicated in activating protein synthesis [Rommel et al., 2001; Glass, 2005].

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INTERLEUKIN-6 (IL-6)

In contrast to IGF-1, the roles of IL-6 in skeletal muscle physiology remains controversial, with the cytokine apparently eliciting both detrimental and beneficial effects on skeletal muscle. It has been shown that prolonged elevations of proinflammatory cytokines (e.g. IL-6) are not only associated with muscle wasting in chronic diseases including HIV AIDS, rheumatoid arthritis and cancer [Tisdale, 2002; Walsmith and Roubenoff, 2002] but also muscle wasting (sarcopenia) that occurs with ageing [Kamel, 2003; Morley and Baumgartner, 2004]. Several studies have been carried out to elucidate the mechanisms by which IL-6 mediates its negative impact on muscle functions both in vivo and in vitro, and it has been reported that IL-6 accelerates protein degradation in skeletal muscle [Strassmann et al., 1992ab; Goodman, 1994; Carson and Baltgalvis, 2010]. Collectively, these data suggest that IL-6 is a potent cytokine that impairs muscle functions and mediates muscle wasting.

In contrast to its negative contribution to skeletal muscle function, in response to intense, prolonged exercise skeletal muscle produces IL-6, which regulates carbohydrate and lipid metabolism, sparing glycogen depletion and increasing satellite cell proliferation (reviewed in [Febbraio and Pedersen, 2002; Pedersen et al., 2004; Pedersen, 2009]). During exercise, contracting skeletal muscle produces IL-6 which induces fat metabolism [van Hall et al., 2003] and glucose uptake by working muscles [Helge et al., 2003] and ultimately it is reported to suppress endotoxemia-mediated TNF-α production [Starkie et al., 2003]. Studies by Wallenius et al. demonstrating that IL-6 knock out mice exhibit obesity and insulin resistance [Wallenius et al., 2002] are also indicative of the important positive metabolic role that IL-6 plays in skeletal muscle function.

INTERACTION OF IGF-1 AND IL-6

Only a few studies have addressed the potential detrimental effects of IL-6 on IGF-1 signalling pathways in skeletal muscle physiology although it is well established that balance between the catabolic effect of cytokines (e.g. IL-6) and anabolic effect of IGF-1 play crucial role in the development of muscle wasting in elderly people (sarcopenia) [Rosen, 2000; Cappola et al., 2003]. These early studies reported that IL-6 inhibits the secretion of IGF-1 and its biological activity. It has been demonstrated that higher plasma IL-6 levels and lower plasma IGF-1 were associated with lower muscle strength and power [Barbieri et al., 2003]. Also there is a growing body of evidence suggesting that IL-6 may interact/interfere with the GH-IGF-1 axis via various signalling cascades [De Benedetti et al., 1997; Lieskovska et al., 2002], including the Janus kinase (JAK) and signal transducer activator of transcription (STAT) pathway, which leads to changes in suppressor of cytokine signalling (SOCS) action, a negative regulator of JAK and STAT signalling [Takahashi et al., 1999; Heinrich et al., 2003]. An alternative study [Lazarus et al., 1993; de Martino et al., 2000] reported that IL-6 inhibited IGF-1 production and its biological activity and that IL-6 overproduction was a mechanism implicated in IGF-1 and IGF-binding protein (IGFBP)-3 downregulation [Lazarus et al., 1993]. Furthermore, NSE/hIL-6 transgenic mouse model illustrated an IGF-1 deficiency and associated growth impairment, which was caused by IL-6-related mechanisms [De Benedetti et al., 1997]. In light of such evidence, biological interactions between IGF-1 and IL-6 can be proposed which may induce a detrimental effect in maintaining an adequate muscle mass and function in elderly people. Therefore, better understanding of such molecular interactions could not only be used to study the skeletal muscle wasting in elderly people but also could lead research into new directions and highlight possible therapeutic strategies for both prevention and treatment of muscle wasting conditions with age and with disease.

We hypothesised that IL-6 blocks IGF-1 activity in skeletal muscle cells where IL-6 levels is high and IGF-1 levels is low (ageing conditions) through blocking its downstream signalling cascades. Our objectives were to investigate (i) The impact of co-incubations of IGF-1 with IL-6 on physiological adaptations of the C2 murine skeletal muscle cell model, (ii) the downstream signalling pathways and (iii) the key myogenic regulatory factors, which may be altered by said incubations. These studies may provide some insight into the underlying cellular mechanisms of impaired muscle mass and function with aging, where IL-6 is elevated and IGF-1 is suppressed [Barbieri et al., 2003; Cappola et al., 2003; Kamel, 2003].

In order to evaluate the combined effects of IGF-1/IL-6 treatments on skeletal muscle cell differentiation and proliferation, we therefore co-cultured C2 cells with IGF-1 (10 ng/ml [Saini et al., 2008]) for 24 h prior to the addition of IL-6 (2.5 ng/ml [Al-Shanti et al., 2008]) to mimic the ageing conditions where low IGF-1 and high IL-6 levels determined [Cappola et al., 2003].

In this study, we demonstrate that IL-6 exerts its detrimental inhibitory effects on IGF-1 activity in skeletal muscle myoblast through blocking its receptor substrate IRS-1 by SOCS-3.

MATERIALS AND METHODS

MATERIALS

All cell and tissue culture media and supplements were purchased as sterile or were filter sterilised through a 0.20 μM filter. Heat-inactivated (hi) foetal bovine serum (FBS) and hi new born calf serum (NCS) were purchased from Gibco (Paisley, Scotland); hi horse serum (HS) was from TCS Biosciences (Corby, England); penstrep (penicillin and streptomycin) and trypsin from Bio Whittaker (Wokingham, England); L-glutamine from BDH (Poole, England), gelatine from Sigma (St. Louis, MO). Plasticware were purchased as sterile from Greiner Bio-one, (Kremsmunster, Austria) unless otherwise stated. Recombinant human IGF-1 and IL-6 were purchased from Bechem (Merseyside, UK), cytometric bead arrays (CBA) from Becton Dickinson (BD Oxford, UK) and creatine kinase (CK) assay kits from Catachem (Bridgeport, CT). All other chemicals, unless otherwise stated, were purchased from Sigma (Poole, Dorset, England).

CELL CULTURE

C2 mouse skeletal myoblasts [Yaffe and Saxel, 1977] were grown in a humidified 5% CO_2 atmosphere at 37°C in growth medium (GM), composed of: DMEM with Glutamax supplemented with 10% FBS, 10% NCS, penstrep and L-glutamine, at final concentrations of

10,000 U/ml and 2 mM, respectively, until 80% confluency was attained. Experiments and differentiation were initiated following washing with phosphate buffered saline (PBS), by transferring to low serum-containing differentiation medium (DM; DMEM plus glutamax, supplemented with 2% HS, pen/strep and L-glutamine (supplemented as above) in the absence or presence of specific cytokines, growth factors and signal inhibitors.

Adherent cells following trypsinisation, and detached cells in the supernatant, were counted using a haemocytometer in the presence of trypan blue dye (Bio Whittaker).

CELL TREATMENTS

Six-well plates were pre-coated with 0.2% gelatine for 5 min at room temperature. Following removal of excess gelatine, cells were seeded at 1×10^5 cells/ml in GM. On attaining approximately 80% confluency, cells were washed twice with PBS and transferred to DM in the absence or presence of either IGF-1 (10 ng/ml upon transfer into DM) or IL-6 (2.5 ng/ml, 24 h post-transfer into DM) or co-incubations of IGF 24 h prior to supplementation with IL-6. For qRT-PCR, morphological and apoptotic studies and CBAs, cells were incubated for a further 48 h following IL-6 administration. For studies involving CK assays, cells were harvested 72 h following IL-6 incubation, as described below. These time points were chosen in order to assess the optimal effects of the triggers on cell proliferation, death and differentiation.

RNA EXTRACTION AND qRT-PCR

Following cell treatments and incubations, differentiation medium was aspirated, cells were washed with PBS and lysed with 200 μl TRIZOL reagent. RNA was extracted from TRIzol homogenates according to the manufacturer's instructions (Invitrogen, CA) and 30 ng of RNA was used per reaction. TaqMan gene expression assays were performed for the target genes (IGF-II, Myogenin and MyoD, SOCS-3 and IRS-1) with StepOnePlus systems (Applied Biosystems, Foster City, CA). TaqMan® RNA-to-C T™ 1-Step Kit with standard TaqMan cycling conditions was used according to the manufacturer's instructions (Applied Biosystems) and all reactions were performed in triplicate. Changes in expression level by Q-PCR were calculated as $-\Delta\Delta$CT [cycle threshold; Livak and Schmittgen, 2001]. The housekeeping gene RNA polymerase II alpha as a reference housekeeping gene (RPIIα) was performed in parallel as control [Radonic et al., 2004].

MORPHOLOGICAL DIFFERENTIATION

Morphological parameters of differentiation, alignment, elongation and fusion were assessed by cell imaging system at 20X magnification at 48 h post IL-6 addition (Leica, DMI 6000 B).

FLOW CYTOMETIC ANALYSIS OF APOPTOSIS AND CYCLIN D1 EXPRESSION

Flow cytomtery is widely used to determine apoptotic population using pre-G1 [Zhang et al., 1999, 2001]. For measuring apoptosis, cells were trypsinised and washed in PBS prior to fixing at −20°C in 75% ethanol. After a minimum of 24 h, cells were washed twice in PBS and resuspended with gentle vortexing in propidium iodide labelling buffer (50 μg/ml propidium iodide, 0.1% sodium citrate, 20 μg/ml ribonuclease A, 0.3% Nonidet P-40, pH 8.3) at approximately $\sim 1 \times 10^6$ cells/ml. For cyclin D1 staining, cells were fixed with 2% formaldehyde and permeabilised with ice-cold 90% methanol. Permeabilised cells were washed twice with PBS and stained with mouse monoclonal FITC-conjugated cyclin D1 antibody (Abcam, Cambridge, UK). The cells were analysed using a Becton Dickinson FACSCalibur flow cytometer. Data were analysed using Cell Quest software (Becton Dickinson, Oxford, England).

CREATINE KINASE ASSAY

Cells were treated as described, washed twice with PBS and lysed in 0.2 ml of 50 mM Tris-MES, pH 7.8, 1% Triton X-100 (TMT buffer). Samples were stored at −80°C, and assayed within 2 weeks of harvesting using a commercially available kit (Catachem CK) according to manufacturer's instructions. The absorbance activity of CK was measured at 3, 4 and 5 min post reaction initiation at a wavelength of 340 nm and changes in absorbance/min were calculated prior to normalisation against total protein content as determined by the BCA™ assay (Pierce, Rockford, IL).

DETERMINATION OF ERK1/2 AND AKT PHOSPHORYLATION

BD™ CBA has been recently validated and used [Krutzik and Nolan, 2003; Krutzik et al., 2004; Schubert et al., 2009; Sharples et al., 2011a] to quantifies multiple proteins, including intracellular phosphorylated signalling proteins, simultaneously. CBA was performed according to manufacturers' instructions. Briefly, after cell treatments, cells were washed at 4°C in PBS, lysed using lysis buffer (1x BD™) and denatured at 100°C. To standardised CBA, protein concentrations were determined as described previously (BCA™ assay), and samples diluted to 15 μg/ml using the assay diluent. Phospho-recombinant protein standards (ERK1/2, and Akt stock 50,000 Units/ml) were prepared using serial doubling dilutions. The relevant capture beads per phosphorylated protein were added to each sample. Following 2 h of incubation, PE detection reagent was added to each tube followed by 1 h incubation. The samples were washed and centrifuged at 300 *g* for 5 min. Cells were then resuspended in 300 μl of fresh wash buffer and analysed using Cell Quest Pro on a BD FACSCalibur. Three hundred events were captured per analyate per sample according to manufacturer's instructions. Data were filtered using FCS Filter™ and analysed using FCAP array software (Both programmes: Hungary Software Ltd., for BD Biosciences, San Jose, CA).

STATISTICAL ANALYSES

All experiments were repeated three times independently in duplicate, unless otherwise stated and were analysed using GraphPad Prism software version 5.0. Results are presented ± standard deviation (SD) of the mean. Statistical significance was determined using one-way ANOVA analysis followed by Tukey-Kramerith multiple post hoc analyses. Results were considered statistically significant when $P < 0.05$ against appropriate controls and marked with * whereas ** denotes ($P < 0.005$), and *** denotes ($P < 0.001$).

RESULTS

C2 myoblasts undergo spontaneous differentiation into myotubes on serum withdrawal, and do not require growth factor addition to stimulate the process [Florini et al., 1991]. Therefore, in this paper, results derived from IGF-1/IL-6 co-treatments were only compared with IGF-1 (10 ng/ml) or IL-6 (2.5 ng/ml) treated cells (control treatments); DM treatment was included in the current study as an internal basal control to validate the IGF-1 and IL-6 effects. The rational for using the above concentrations of IGF-1 and IL-6 which were previously established by our laboratory [Al-Shanti et al., 2008; Saini et al., 2008] is to mimic the in vivo aging conditions which is associated with reduced circulating IGF-1and increased levels of IL-6 [Cappola et al., 2003].

IMPACT OF IGF-1/IL-6 ON MYOBLASTS MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

Morphological studies were performed (48 h following dosing the cells with IL-6) to examine whether co-culture of the myoblasts with IGF-1 and IL-6 may negatively impact on IGF-1 action. Morphological differentiation was assessed across several random microscopic fields/treatment regimes. Myoblasts in DM or IGF-1 alone showed the most prominent differentiation where cell fusion and large myotubes formation are evident (Fig. 1) whereas limited myotube formation is observed in cells cultured with IL-6 (moderate differentiation). However cells co-incubated with IGF-1/IL-6 did not exhibit any apparent myoblast fusion, myotube formation or differentiation characteristics (Fig. 1A).

To further confirm differentiation blockade, biochemical studies using CK as a marker of differentiation were performed (72 h following dosing with IL-6). Confirming the morphological observations, CK activity was reduced significantly where the differentiation is blocked ($P < 0.005$) in the cells treated with IGF-1/IL-6 compared to control treatments. CK activity in co-incubation treatments was (941 ± 150 mU/mg/ml) relative to cells treated with IGF-1 (1,879 ± 200 mU/mg/ml, $P < 0.001$) or DM (1,550 ± 260 mU/mg/ml, $P < 0.05$) or IL-6 (1,500 ± 170 mU/mg/ml, $P < 0.05$) alone (Fig. 1B).

IMPACT OF IGF-1/IL-6 ON SKELETAL MYOBLAST DEATH AND SURVIVAL

While we were assessing the morphological differentiation, a high proportion of cell death was observed in myoblasts treated with IGF-1/IL-6. We therefore investigated further the effects of co-cultures on myoblast death and survival using flow cytometry [Zhang et al., 1999]. As expected following morphological assessment, a significant increase ($P < 0.001$) in the pre-G1 apoptotic peak (22.3 ± 0.9%) was evident in the cells treated with IGF-1/IL-6 compared to cell death levels in IGF-1 (14 ± 2.9%, $P < 0.001$) and IL-6 (12 ± 0.8%, $P < 0.001$) treatment alone (Fig. 2A). These findings were further confirmed by determining the expression level of cell cycle marker cyclin D1 protein [Cheng et al., 1998]. Co-treatments induced dramatic reduction in cyclin D1 expression levels (7 ± 3.6%) where the cells death was high relative to the control treatments. However, the level of cyclin D1 expressions was high in the cells treated with IGF-1 (34 ± 4.2%, $P < 0.001$) and DM (22 ± 2%, $P < 0.005$) where the progression in cell cycle was high compared to IGF-1/IL-6 treated cells (Fig. 2B).

Previous studies have demonstrated that IGF-II is a potent autocrine survival and differentiation mediator for skeletal myoblasts [Stewart and Rotwein, 1996]. To address whether altered IGF-II expression underpins the reduction in differentiation and high levels of cell death in the presence of IGF/IL-6, expression levels of this gene were determined using qRT-PCR. IGF-II expression was indeed significantly suppressed in IGF-1/IL-6 treated myoblasts by (0.07-fold ± 0.01), relative to IGF-1 and IL-6 treated cells, respectively (1.2-fold ± 0.23; $P < 0.001$ and 0.70-fold ± 0.25; $P < 0.05$, Fig. 2C).

IMPACT OF IGF-1/IL-6 ON MYOGENIC FACTORS mRNA EXPRESSIONS

Myogenin and MyoD are potent myogenic factors that regulate myoblast differentiation [Venuti and Cserjesi, 1996]. Therefore to examine whether co-incubation of myoblasts with IGF-1/IL-6 altered their expression, in association with reduced differentiation and reduced IGF expression, the mRNAs expression levels of both genes was determined by qRT-PCR. The mRNA levels of myogenin and MyoD were extremely reduced by 90% and 70%, respectively, in the co-incubation treatment relative to control cell treatments. Co-administration of myoblasts with IGF-1/IL-6 decreased expression levels of myogenin significantly (0.05-fold ± 0.005, $P < 0.005$) when compared to DM (1.0-fold ± 0.2) or IGF-1 (1.3-fold ± 0.05) alone treatment (Fig. 3A). Similarly, co-incubation treatment showed significant reduction in the MyoD mRNA levels (0.27-fold ± 0.028, $P < 0.05$) relative to DM (1.0-fold ± 0.14), IGF-1 (1.1-fold ± 0.2) and IL-6 (0.9-fold ± 0.06) treatments (Fig. 3B).

IMPACT OF IGF-1/IL-6 ON ERK AND AKT SIGNALLING PATHWAYS

We next investigated the potential signalling pathways by which IL-6 in conjunction with IGF-1 may manipulate myoblast survival and differentiation. CBA were used to quantify the levels of relevant phosphorylated proteins [Krutzik and Nolan, 2003; Krutzik et al., 2004; Schubert et al., 2009; Manjavachi et al., 2010] involved in downstream signalling of the IGF-1 receptor, including Akt and ERK1/2. ERK1/2 phosphorylation levels were significantly ($P < 0.05$) suppressed in myoblasts treated with IGF-1/IL-6 (44 U/ml ± 3) compared to the single treatment with IGF-1 (55 U/ml ± 3, $P < 0.005$) or IL-6 (52 U/ml ± 2.3; $P < 0.05$, Fig. 4A). However, Akt phosphorylation levels were only significantly decreased in the cells treated with IGF-1/IL-6 (14 U/ml ± 2.5) relative to control treatment IGF-1 control (22 U/ml ± 2.0; $P < 0.05$, Fig. 4B).

IMPACT OF IGF-1/IL-6 ON SOCS-3 AND IRS-1 mRNA EXPRESSIONS

IGF-1 induces differentiation and proliferation through the activation of its receptor and IRS-1 which activates the downstream signalling pathways ERK1/2 and PI3KAkt [Coolican et al., 1997].

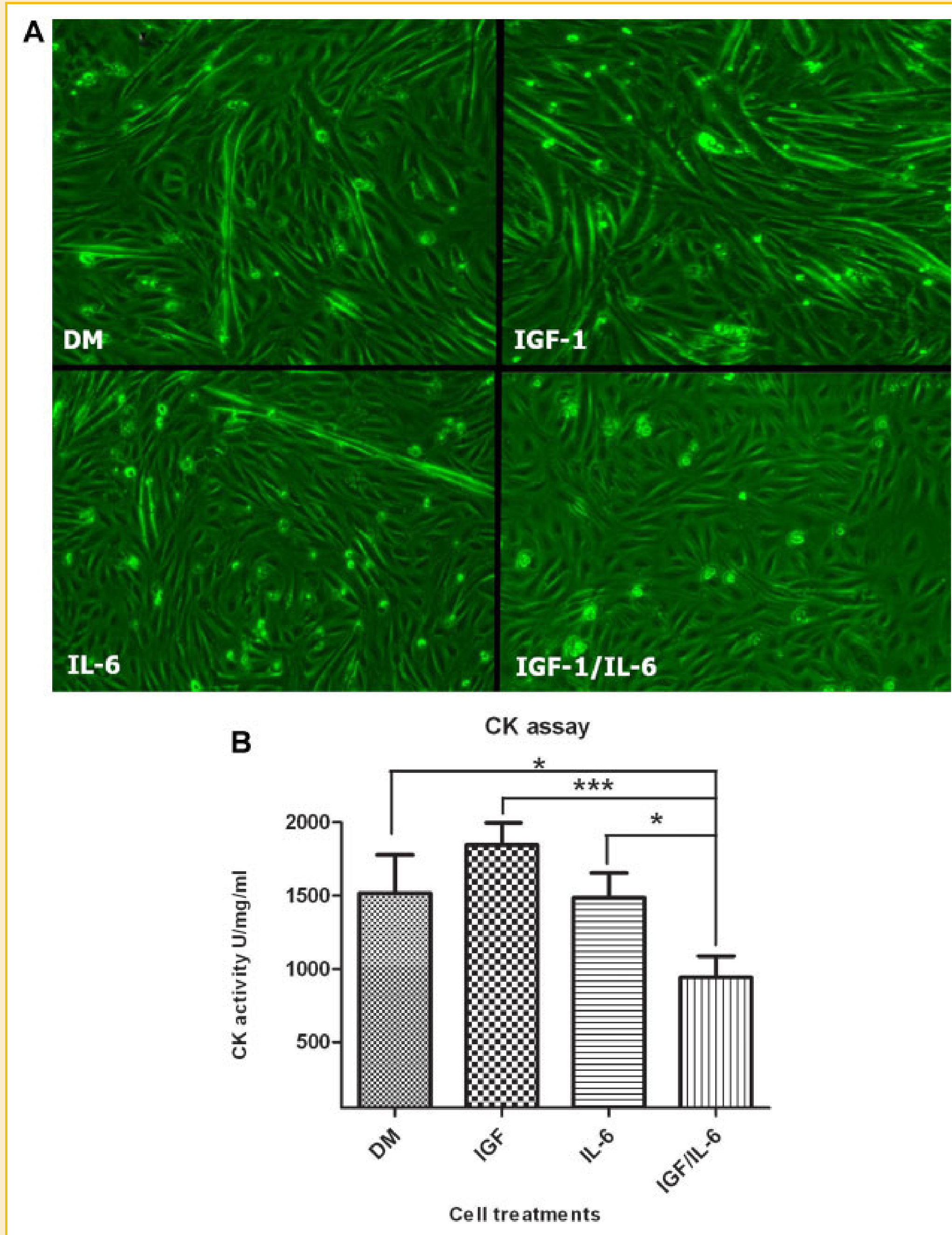

Fig. 1. Effects of IGF-1/IL-6 on C2 myoblasts morphological and biochemical characteristics. C2 myoblasts were cultured with DM only or DM supplemented with 10 ng/ml IGF-1, 2.5 ng/ml IL-6 or IGF-1/IL-6. At the end of cell treatments (48 h after adding IL-6), cells were subjected to morphological (A) and biochemical (B) studies. A: DM or IGF-1 alone showed the most prominent differentiation where cell fusion and large myotubes formation are evident. In contrast, cells co-incubated with IGF-1/IL-6 did not exhibit any apparent myoblast fusion, myotube formation or differentiation characteristics. B: CK activity (a biochemical marker of differentiation) was reduced significantly where the differentiation is blocked in myoblasts treated with IGF-1/IL-6 compared to control treatments. Image in (A) is a representative photomicrograph of random fields from four independent experiments whereas data in (B) represent the mean $\pm$ SD of representative of four experiments in duplicate. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Our present data showed that co-incubation of the myoblasts with IGF-1/IL-6 not only blocks differentiation and proliferation but also the relevant signalling pathways were also inhibited. All these findings promoted us to investigate IRS-1, the key central molecular which is involved in both signalling pathways of IGF-1 (Fig. 5A). Interestingly, in myoblasts co-cultured with IGF-1/IL-6, IRS-1 mRNA was significantly reduced (0.65-fold $\pm$ 0.13) compared to IGF-1 (1.2-fold $\pm$ 0.13, $P < 0.005$) and DM (1.0-fold $\pm$ 0.15, $P < 0.05$) control treatments whereas no significant reduction was observed relative to IL-6 (0.95-fold $\pm$ 0.15). IL-6 signalling pathway is know to induce SOCS-3 mRNA expression levels [Lieskovska et al., 2003]. In addition, SOCS-3 is suggested to negatively control

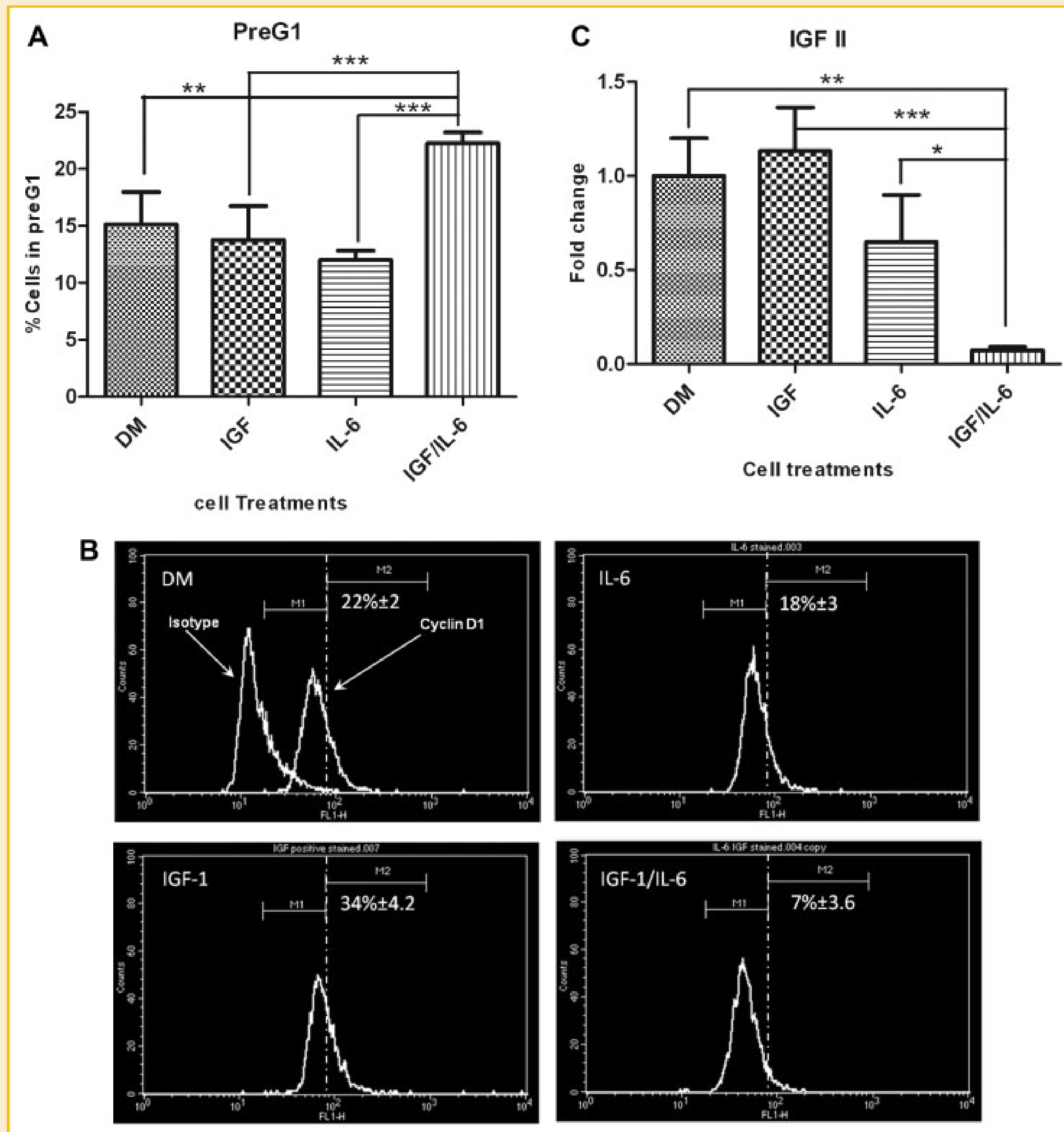

Fig. 2. Effect of IGF-1/IL-6 on C2 myoblasts death and survival. C2 myoblasts were cultured with DM only or DM supplemented with 10 ng/ml IGF-1, 2.5 ng/ml IL-6 or IGF-1/IL-6 (IL-6 was added 24 h after dosing the cells with IGF-1). At the end of cell treatments (48 h after adding IL-6), cells were subjected to flow cytometry analysis for Pre-G1 (apoptosis marker) (A) and cyclin D1 determination (cell cycle marker) (B) and for qRT-PCR to quantify mRNA levels of IGF-1I (a survival marker) (C). Pre-G1 apoptotic peak was significantly evident in the cells treated with IGF-1/IL-6 (22 ± 1%) relative to cell control treatments (A). IGF-1/IL-6 showed marked inhibition (7 ± 4.2%) of cyclin D1 compared with controls confirming pre-G1 data (B). Myoblasts survival factor mRNA IGF-II was extremely reduced (0.07 ± 0.017) as a result of IGF-1/IL-6 treatment relative to either single treatment (C). Data represent the mean ± SD of representative of four experiments in duplicate.

the signalling pathways of IGF-1 through binding to its receptor or IRS-1 [Mooney et al., 2001]. We therefore tested whether IGF-1/IL-6 treatments affects the expression levels of SOCS-3. Co-treatments of myoblasts with IGF-1/IL-6 induced the expression of SOCS-3 mRNA by 1.5-fold ± 0.08 over control treatment DM (1.0-fold ± 0.09, $P < 0.005$) and IGF-1 (0.9-fold ± 0.16, $P < 0.005$), while there was no significant difference between cells treated with IL-6 and IGF-1/IL-6 (Fig. 5B).

DISCUSSION

To date, most researchers have focused on investigating the impact of IGF-1 or IL-6 alone on skeletal muscle metabolism, hypertrophy or atrophy and speculate as to potential interactive effects. Given their potential for cross-talk, the purpose of the study described herein was to gain insight into the mechanisms by which IL-6 may modulate the biological functions of IGF-1. The results presented

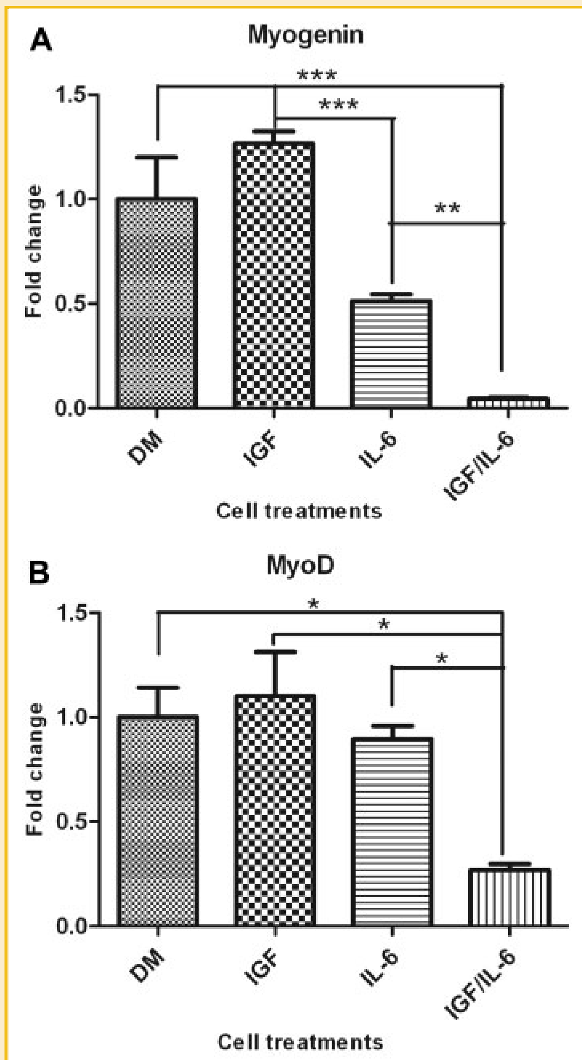

Fig. 3. Effect of IGF-1/IL-6 on myogenic factor mRNA expressions. C2 myoblasts were cultured with DM only or DM supplemented with 10 ng/ml IGF-1, 2.5 ng/ml IL-6 or IGF-1/IL-6 (IL-6 was added 24 h after dosing the cells with IGF-1). At the end of cell treatments (48 h after adding IL-6), total RNA was extracted from cells, and 30 ng from each sample was used to perform real time qRT-PCR. Changes in expression level of target genes mRNAs Myogenin (A) and MyoD (B) by Q-PCR were calculated as $-\Delta\Delta CT$. The housekeeping gene RPIIα was performed in parallel as control. The mRNA levels of myogenin and MyoD were dramatically reduced by 90% and 70%, respectively, in the co-incubation treatment relative to control cell treatments. Data represent the mean $\pm$ SD of representative of four experiments in duplicate, with all samples being run in triplicate for RT-PCR.

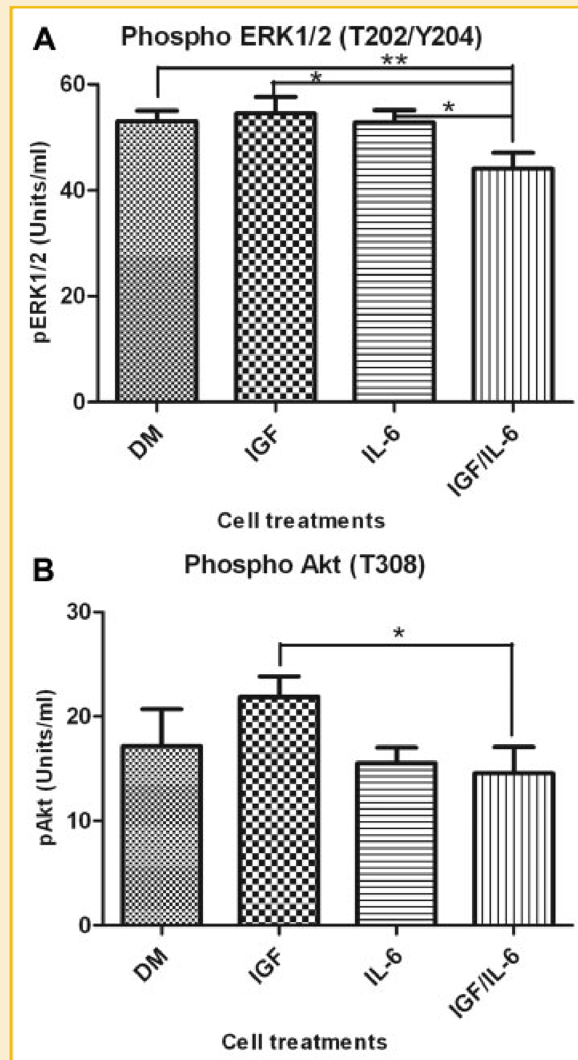

Fig. 4. Effect of IGF-1/IL-6 on phosphorylation levels of ERK1/2, and Akt. C2 myoblasts were cultured with DM only or DM supplemented with 10 ng/ml IGF-1, 2.5 ng/ml IL-6 or IGF-1/IL-6 (IL-6 was added 24 h after dosing the cells with IGF-1). At the end of cell treatments (48 h after adding IL-6), 15 μg/ml of total protein lysate was used to quantify the phosphorylated protein levels of ERK1/2 (A), and Akt (B) using BD™ CBA. ERK1/2 phosphorylation levels were significantly suppressed in myoblasts treated with IGF-1/IL-6 ($20 \pm 3\%$) compared to the single treatment. However, Akt phosphorylation levels were only significantly decreased in the cells treated with IGF-1/IL-6 ($35 \pm 2.5\%$) relative to IGF-1 control treatment. These data present the mean $\pm$ SD of the treatments performed three times in triplicate.

here, provide important evidence concerning the negative impact of IL-6 on IGF-1 function in skeletal muscle myoblasts and extend previous findings illustrating that IL-6 can negatively interfere with the IGF-1 axis [De Benedetti et al., 1997; Barbieri et al., 2003; Laviola et al., 2008; Perrini et al., 2010].

Herein, we demonstrated that morphological differentiation of myoblasts into myotubes was blocked in the cells co-incubated with IGF-1/IL-6 using relatively high concentration of IL-6 (2.5 ng/ml) and low concentration of IGF-1 (10 ng/ml) to mimic the in vivo ageing conditions. This suppression of differentiation was also associated with a significant decrease in CK activity in the cells treated with IGF-1/IL-6 in the face of increased apoptosis. These data were also associated with a significant increase in pre-G1 positive apoptotic cells (22%). The co-incubation of IGF-1/IL-6 not only resulted in reduced differentiation but increased apoptotic cell death in the face of decreased cyclin D1 protein accumulation—a parameter not seen with either treatment alone. Given the reduction in differentiation and the increase in myoblasts death, we assessed

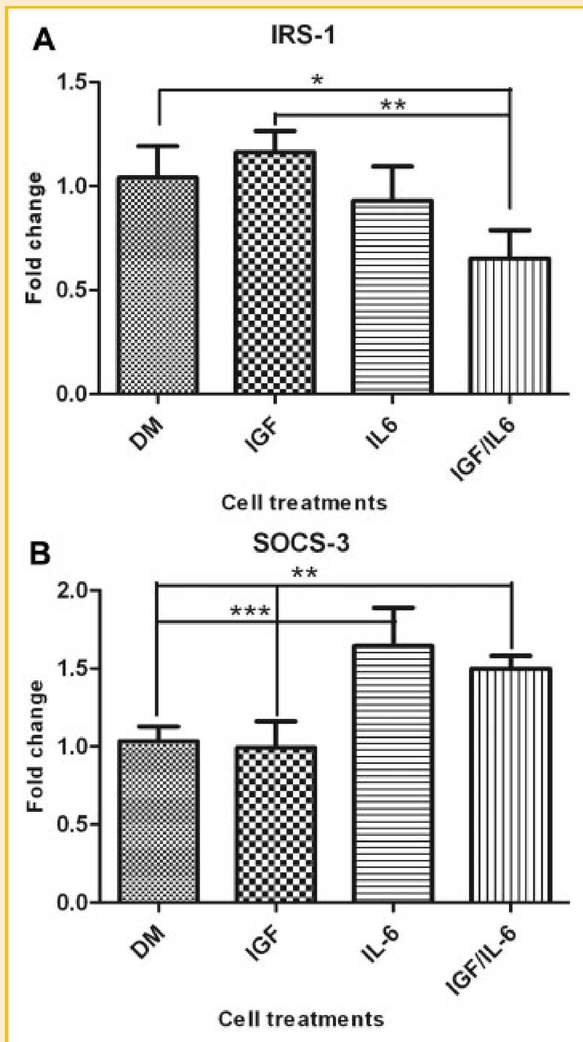

Fig. 5. Effect of IGF-1/IL-6 on IRS-1 and SOCS-3 mRNA expressions. C2 myoblasts were cultured with DM only or DM supplemented with 10 ng/ml IGF-1, 2.5 ng/ml IL-6 or IGF-1/IL-6 (IL-6 was added 24 h after dosing the cells with IGF-1). At the end of cell treatments (24 h after adding IL-6), total RNA was extracted from cells, and 30 ng from each sample was used to perform real time qRT-PCR. Changes in expression level of target genes mRNAs IRS-1 (A) and SOCS-3 (B) by Q-PCR were calculated as $-\Delta\Delta CT$. The housekeeping gene RPIIα was performed in parallel as control. Co-treatments with IGF-1/IL-6 significantly reduced the IRS-1 mRNA by ~35% relative to control treatments (A) whereas SOCS-3 mRNA was enhanced by ~50% compared to DM and to IGF-1 control treatments (B).

the expression levels of IGF-II, a known survival and differentiation factor [Stewart and Rotwein, 1996; Yoshiko et al., 2002]. Consistent with the result of above studies, which reported low expression levels of IGF-II in C2 myoblasts where differentiation was reduced and death was increased, we detected low expression levels of IGF-II in the myoblasts co-administrated with IGF-1/IL-6.

It is widely established that MyoD and myogenin are essential myogenic mediators during the transition from proliferation to differentiation of skeletal muscle cells [Florini et al., 1996; Coolican et al., 1997]. A number of studies have reported that IL-6 may alter/interfere with the IGF-1 axis through the alteration not only of several signalling cascades (reviewed in Ref. [Perrini et al., 2010]), but also through a specific down-regulation of the expression of myogenic regulatory proteins MyoD and myogenin [Langley et al., 2002; McCroskery et al., 2003]. Our data indeed illustrate that the co-incubation of IGF-1/IL-6 culminates in reduced differentiation and significant reductions in the expression of both myoD and myogenin. This raised the need to ascertain what signalling pathways may be activated or inhibited to enable the detrimental effects of IGF-1/IL-6 on myoblast survival and differentiation.

A possible signalling mechanisms by which IL-6 may negatively affect IGF-1-mediated survival and differentiation are through the negative modulation of its pro-survival, pro-differentiation signalling pathways including IRS-1/PI3K/Akt [Lazarus et al., 1993; McCroskery et al., 2003; Kim et al., 2004; Adams and Vaziri, 2006] and MAPKs (p38, ERK1/2 and JNK) [Laviola et al., 2008]. In our signalling pathways studies, IGF-1 did not show a robust effect on the phosphorylation levels of ERK1/2 and Akt. This unexpected effects could be explained by the findings that under basal condition of differentiation, we and others reported [Florini et al., 1991; Saini et al., 2008; Sharples et al., 2011b] an increase in ERK and Akt phosphorylation which could explain the comparable levels of these proteins (ERK1/2 and Akt) in myoblasts cultured with DM only and with IGF-1 treated cells. In addition, IGF-1 did not induce dramatic effect on phosphorylation levels of these proteins may due to the low IGF-1 concentration (10 ng/ml) used and late time points of determining these proteins (IGF-1 effects could be lost at late time-point 48 h). Importantly, in these studies, we determined that ERK1/2 and Akt were all altered, with significant down-regulation measured in the C2 myoblasts co-administrated with IGF-1/IL-6. Suppression of Akt signalling pathways may underpin increase in apoptosis as well as the inhibition of MyoD and myogenin expression which ultimately leads to reduced differentiation, whereas inhibition of ERK1/2 may result in impaired growth and also reduced differentiation as we have previously reported that ERK [Foulstone et al., 2004] is important regulators of differentiation. Moreover, ERK1/2 signalling pathway is know to induce cell cycle with cooperative interactions with PI3K/Akt through the activation of the cell cycle mediator cyclin D1 [Gille and Downward, 1999; Kuemmerle et al., 2004]. This data may support and explain the reduced levels of cyclin D1 in the myoblasts cultured with IGF-1/IL-6 where the cell death enhanced and differentiation blocked over the controls.

Unexpected inhibition of ERK1/2 and Akt cascades in co-treatments was also explained by the reduced levels of key molecule IRS-1 which mediates both signalling pathways in myoblasts [Coolican et al., 1997]. Considering the biological evidence which showed that IGF-1 and IL-6 signalling pathways could be regulated by SOCS-3 through their downstream targets [Mooney et al., 2001; Alexander, 2002], we determined the expression levels of SOCS-3 mRNA. Consistent with above findings, we found that SOCS-3 mRNA levels was elevated in myoblasts treated with IGF-1/IL-6. This observations is supported by previous data in which SOCS-3 was identified as a negative regulator for IGF-1 cascade [Mooney et al., 2001]. Collectively, these findings may provide important evidence regarding the negative interaction of IL-6 with IGF-1 in terms of muscle cell survival and differentiation.

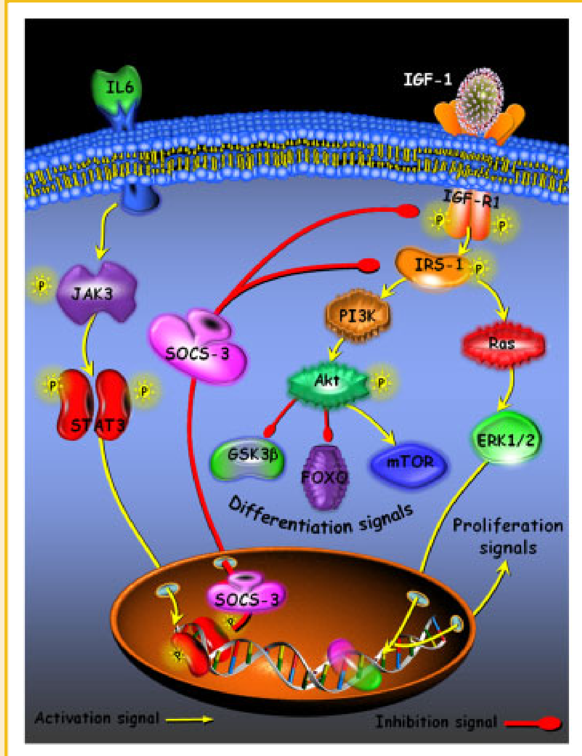

Fig. 6. Schematic diagram of the proposed inhibitory signalling pathway involved in IGF-1 and IL-6 interactions. Binding of IL-6 to its receptor in myoblasts triggers several intracellular signalling cascades that ultimately induce SOCS-3 expression. Upon activation, SOCS-3 is translocated from nucleus to cytoplasm where it may interact/bind either with IGF-1R or IRS-1 blocking their phosphorylation. Inhibitions of such central signalling molecules suppress downstream signalling cascades. As a consequence to this inhibition, downstream signalling pathways of IGF-1 (ERK1/2 activates proliferation and PI3K induces differentiation [Coolican et al., 1997]) will be blocked which leads to the observed inhibitory effects of IL-6 on IGF-1 functions (adapted from [Saini et al., 2006; Al-Shanti and Stewart, 2009]).

CONCLUSIONS

Although, it is well established that prolonged elevations of proinflammatory cytokines (e.g. IL-6) and low levels of circulating anabolic cytokines (e.g. IGF-1) are associated with muscle wasting that occurs during the sarcopenia of ageing [Kamel, 2003; Morley and Baumgartner, 2004; Haddad et al., 2005], the mechanisms underpinning the cross-talk between IGF-1 and IL-6 have not been well elucidated. While, our data do not yet provide the full mechanisms whereby IL-6 negatively interacts with IGF-1, they do provide new insights into the potential for cross-talk between the two myokines and propose a mechanism by which IL-6 impairs IGF-1 function summarised in Figure 6. Importantly, these data have implications with regard to the interactions of these key molecules in regulating muscle mass and raise important issues of using hypertrophic agents such as IGF to counteract atrophic situations where IL-6 may be elevated. Finally, it will be interesting to examine (i) how SOCS-3 interacts with IRS-1? (ii) whether SOCS-3 interacts only with IRS-1 to induce the inhibitory effects or other molecules are involved.

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