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Insulin-like growth factor-I inhibits dexamethasone-induced proteolysis in cultured L6 myotubes through PI3K/Akt/GSK-3β and PI3K/Akt/mTOR-dependent mechanisms☆

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Abstract

We and others reported previously that IGF-I inhibits dexamethasone-induced proteolysis in cultured L6 myotubes. Recent evidence suggests that this effect of IGF-I at least in part reflects PI3K/Akt-mediated inhibition of Foxo transcription factors. The potential role of other mechanisms, downstream of PI3K/Akt, is not well understood. Here we tested the hypothesis that PI3K/Akt-mediated inactivation of GSK-3β and activation of mTOR contribute to the anabolic effects of IGF-I in dexamethasone-treated myotubes. Cultured L6 myotubes were treated with 1 μM dexamethasone in the absence or presence of 0.1 μg/ml of IGF-I and inhibitors of GSK-3β and mTOR. Protein degradation was measured by determining the release of trichloroacetic acid soluble radioactivity from myotubes that had been prelabeled with ^{3}H-tyrosine for 48 h. IGF-I reduced basal protein breakdown rates and completely abolished the dexamethasone-induced increase in myotube proteolysis. These effects of IGF-I were associated with increased phosphorylation of Akt, GSK-3β, and the mTOR downstream targets $p70^{S6K}$ and 4E-BP1. The PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin reversed the anabolic effect of IGF-I in dexamethasone-treated myotubes. In addition, the GSK-3β inhibitors LiCl and TDZD-8 reduced protein degradation in a similar fashion as IGF-I. Our results suggest that PI3K/Akt-mediated inactivation of GSK-3β and activation of mTOR contribute to the anabolic effects of IGF-I in dexamethasone-treated myotubes.

Keywords: L6 myotubes; Dexamethasone; Proteolysis; IGF-I; Cell signaling

1. Introduction

Muscle wasting induced by sepsis, burn injury, cancer, and a number of other catabolic conditions, mainly reflects stimulated protein breakdown, in particular myofibrillar protein breakdown (Fang, James, Fischer, & Hasselgren, 1995; Hasselgren & Fischer,

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2001; Lecker et al., 2004). Although multiple factors may regulate muscle proteolysis, there is evidence that glucocorticoids play a predominant role in muscle wasting conditions (Hasselgren, 1999). In previous studies, treatment of cultured myotubes with dexamethasone resulted in increased protein degradation and atrophy, supporting the role of glucocorticoids in muscle catabolism (Hong & Forsberg, 1995; Wang, Luo, Wang, & Hasselgren, 1998).

IGF-I has a nitrogen sparing effect in normal and catabolic conditions (Cioffi et al., 1994) and this effect of IGF-I is, at least in part, caused by inhibited muscle protein degradation (Fang, Li, Wang, Fischer, Hasselgren, 1997, 1998; Fang, Li, Wray, & Hasselgren, 2002). We and others reported recently that IGF-I inhibits dexamethasone-induced proteolysis in cultured muscle cells (Li, Hasselgren, Fang, & Warden, 2004; Sacheck, Ohtsuka, McLary, & Goldberg, 2004). Other studies suggest that PI3K/Akt-mediated inhibition of Foxo transcription factors is an important mechanism of the anabolic effects of IGF-I in skeletal muscle (Sandri et al., 2004; Stitt et al., 2004). In contrast, the potential role of other PI3K/Akt downstream mechanisms for the inhibitory effects of IGF-I on muscle protein breakdown are less well understood. In the present study, we tested the hypothesis that PI3K/Akt-mediated inactivation of GSK-3β and activation of mTOR contribute to the anabolic effects of IGF-I in dexamethasone-treated myotubes.

2. Materials and methods

2.1. Materials

Tissue culture medium components and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY). Recombinant human IGF-I was a gift from Genentech (South San Francisco, CA). Dexamethasone was purchased from Sigma (St. Louis, MO). L-[ring-3,5-^{3}H]-tyrosine was from Perkin Elmer Life Science (Boston, MA). The signaling pathway inhibitors LY294002, rapamycin, PD98059 and thiadiazolidinone (TDZD)-8 were obtained from Calbiochem (San Diego, CA). Lithium chloride (LiCl) was from Sigma. Protein assay and Western blotting reagents were from Bio-Rad Laboratories (Hercules, CA). All antibodies (rabbit polyclonal) were purchased from Cell Signaling Technology (Beverly, MA): against mouse Akt and phospho (Ser473)-Akt; against human GSK-3β and phospho (Ser9)-GSK-3β; against human p70^{S6k} and phospho (Thr389)-p70^{S6k}; against human 4E-BP1 and mouse phospho (Ser65)-4E-BP1; against rat p44/42 MAP kinase and against human phospho (Thr202/Tyr204)-p44/42 MAP kinase; against human p38 MAP kinase and phospho (Thr180/Tyr182)-p38 MAP kinase. Horse radish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) was used as secondary antibody. Enhanced chemiluminescence (ECL) immunoblotting detection reagent was from Amersham Biosciences (Piscataway, NJ).

2.2. Cell culture

L6 rat skeletal muscle cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) as described in detail previously (Li et al., 2004). When the cells were approximately 80% confluent, they were seeded in 24-well culture plates (2.5×10^4 cells/well) for measurement of protein degradation or in 10-cm dishes (2.5×10^5 cells) for Western blot analysis. Once the cells achieved confluence, the concentration of FBS was reduced to 2% to induce differentiation into myotubes. All experiments were performed in differentiated myotubes.

2.3. Measurement of protein degradation

After differentiation into myotubes, the cells were labeled with 1 μCi of L-[ring-3,5-^{3}H]-tyrosine/ml for 48 h in DMEM containing 2% FBS. The myotubes were then washed, fresh medium was added, and protein degradation was measured by determining the release of trichloroacetic acid (TCA)-soluble radioactivity during 6 h and expressed as the percentage protein degraded as described in detail previously (Wang et al., 1998). During this 6 h period, myotubes were treated with 1 μM dexamethasone in the absence or presence of IGF-I. In most of the experiments, IGF-I was added to the myotubes at a concentration of 0.1 μg/ml. In one experiment, an IGF-I concentration of 1 μg/ml was used. The IGF-I concentration used in the different experiments is indicated in the figures. To assess the role of different signaling pathways, inhibitors of individual pathways were added to the myotubes 1 h before

the addition of dexamethasone and IGF-I. The concentrations of the inhibitors are described in Section 3.

2.4. Western blot analysis

After different treatments, the myotubes were harvested and lysed in ice-cold lysis buffer as described in detail previously (Luo, Hershko, Robb, Wray, & Hasselgren, 2003). All manipulations of cell lysates were performed at 4 °C. After centrifugation for 10 min at 10,000 rpm, protein concentration in the supernatants was determined as described by Bradford (1976) using bovine serum albumin as standard. Aliquots of myotube extracts containing 50 μg of protein were suspended in Laemmli sample buffer with 5% 2-mercaptoethanol and boiled for 3 min. Proteins were separated by electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dried milk and incubated overnight at 4 °C with primary antibody followed by incubation with secondary antibody. A molecular weight marker (Kaleidoscope Prestained Standards, Bio-Rad Laboratories) was included in the experiments. The immunoblotted proteins were detected using enhanced chemiluminescence reagents and the membranes were exposed on radiographic film (Eastman Kodak, Rochester, NY).

2.5. Statistics

Results are presented as mean ± S.E.M. Analysis of variance followed by Tukey's test was used for statistical analysis; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Protein degradation in L6 myotubes is increased by dexamethasone and inhibited by IGF-I

Treatment of cultured L6 myotubes with 1 μM dexamethasone for 6 h resulted in an approximately 20% increase in protein degradation (Fig. 1). This result is consistent with previous reports (Hong & Forsberg, 1995; Wang et al., 1998) and supports the concept that glucocorticoids are an important regulator of muscle protein breakdown (Hasselgren, 1999). When the myotubes were treated with 0.1 μg/ml of IGF-I, basal protein degradation rate was reduced by approximately 20% and the dexamethasone-induced increase in protein breakdown was completely abolished (Fig. 1), confirming a recent report from our laboratory (Li et al., 2004). The concentration of IGF-I used here was based on results from preliminary experiments in which 0.1 μg/ml of IGF-I caused a maximal inhibition of protein degradation in L6 myotubes. In those experiments, protein degradation in dexamethasone-treated myotubes was reduced by approximately 5, 20, and 30% at the IGF-I concentrations 0.001, 0.01, and 0.1 μg/ml, respectively. No further inhibition of protein degradation was observed when the myotubes were treated with 1 or 10 μg/ml of IGF-I. In most subsequent experiments, an IGF-I concentration of 0.1 μg/ml was used.

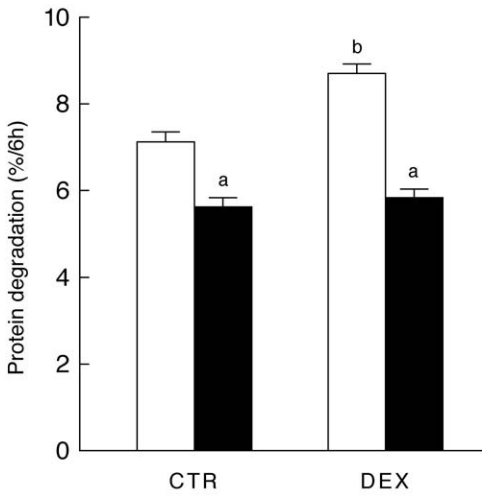

Fig. 1. The effects of dexamethasone and IGF-I on protein degradation in cultured L6 myotubes. Myotubes were cultured for 6 h in the absence (CTR) or presence of 1 μM dexamethasone (DEX) with (filled bars) or without (open bars) 0.1 μg/ml of IGF-I. Protein degradation was determined by measuring the release of TCA-soluble radioactivity from proteins that had been prelabeled with ^{3}H-tyrosine as described in Section 2. Results are means ± S.E.M. with $n = 6$ for each group. (a) $p < 0.05$ vs. no IGF-I; (b) $p < 0.05$ vs. all other groups by ANOVA.

3.2. IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes is associated with activation of PI3K/Akt and is inhibited by LY294002

In order to examine the role of the PI3K/Akt signaling pathway in the effect of IGF-I on dexamethasone-

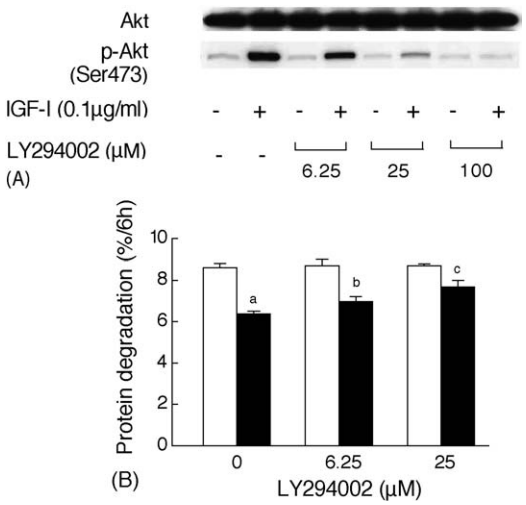

Fig. 2. (A) The effects of IGF-I and LY294002 on the expression of total and phosphorylated Akt in dexamethasone-treated cultured L6 myotubes. Myotubes were cultured for 6 h in the presence of 1 μM dexamethasone and in the absence or presence of 0.1 μg/ml IGF-I or different concentrations of LY294002 as indicated in the figure. The blots are representative of 4 repeated blots. (B) The effects of IGF-I and LY294002 on protein degradation in dexamethasone-treated myotubes. Myotubes were treated with 0.1 μg/ml of IGF-I (filled bars) in the presence of different concentrations of LY294002 as indicated in the figure. Results are means ± S.E.M. with $n = 6$ in each group. (a–c) $p < 0.05$ vs. corresponding no IGF-I (open bars); (a–c) $p < 0.05$ vs. each other.

induced proteolysis, we first determined the levels of phosphorylated Akt (p-Akt) in the myotubes. Addition of IGF-I to dexamethasone-treated myotubes increased p-Akt levels (Fig. 2A) suggesting that the IGF-I-induced inhibition of protein breakdown was accompanied by activation of PI3K/Akt signaling. Next, we tested whether inhibition of PI3K would influence the anti-catabolic effect of IGF-I in dexamethasone-treated myotubes. This was accomplished by treating the myotubes with the PI3K inhibitor LY294002. As expected, the IGF-I-induced increase in p-Akt levels was inhibited by LY294002 in a dose-dependent fashion (Fig. 2A). Importantly, the IGF-I-induced inhibition of proteolysis was also reversed by LY294002 in a dose-dependent fashion (Fig. 2B). In additional experiments (unpublished), we found that concentrations of LY294002 greater than 25 μM caused non-specific effects on myotube protein degradation; 25 μM LY294002 was therefore the highest concentration used here when protein degradation was measured.

3.3. Inhibition by IGF-I of protein degradation in dexamethasone-treated myotubes is associated with phosphorylation (inactivation) of GSK-3β

Activation of the PI3K/Akt signaling pathway results in downstream phosphorylation (inactivation) of GSK-3β. The next set of experiments was designed to test the role of GSK-3β phosphorylation (inactivation) in the anti-catabolic effects of IGF-I in dexamethasone-treated myotubes. First, we measured the levels of phosphorylated GSK-3β (p-GSK-3β) in dexamethasone-treated myotubes exposed to IGF-I. Treatment of the myotubes with IGF-I resulted in increased phosphorylation of GSK-3β and this effect of IGF-I was inhibited by LY294002 in a dose-dependent fashion (Fig. 3A). This result suggests that inhibition of protein degradation by IGF-I in dexamethasone-treated myotubes is associated with PI3K-dependent phosphorylation (inactivation) of GSK-3β.

In order to further test the role of GSK-3β inhibition on myotube protein degradation, dexamethasone-treated myotubes were next exposed to one of the GSK-3β inhibitors, LiCl or TDZD-8 (Ryves & Harwood, 2001; Martinez, Alonso, Castro, Perez, Moreno, 2002). Treatment with either drug resulted in a dose-dependent inhibition of protein degradation (Fig. 3B and C) and at the highest concentrations, LiCl and TDZD-8 caused a similar inhibition of protein degradation as observed following treatment of the myotubes with IGF-I (compare with Fig. 1). Furthermore, addition of 20 mM LiCl or 10 μM TDZD-8 to myotubes treated with 0.1 μg/ml of IGF-I did not reduce protein degradation beyond the inhibition already caused by IGF-I (data not shown). Taken together, the results shown in Fig. 3 suggest that IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes may at least in part reflect inhibition of GSK-3β.

In order to test whether LiCl and TDZD-8 inhibit GSK-3β activity by phosphorylation of the kinase, p-GSK-3β levels were determined. Treatment of the myotubes with LiCl increased p-GSK-3β levels (Fig. 4A), possibly by activating PI3K/Akt signaling (Chalecka-Franaszek & Chuang, 1999). Thus, LiCl may inhibit GSK-3β activity at least in part by phosphorylating the kinase, although other mechanisms are probably involved as well (Ryves & Harwood, 2001). In contrast, TDZD-8 did not increase GSK-3β phosphorylation (Fig. 4B), suggesting that TDZD-8 inhibits

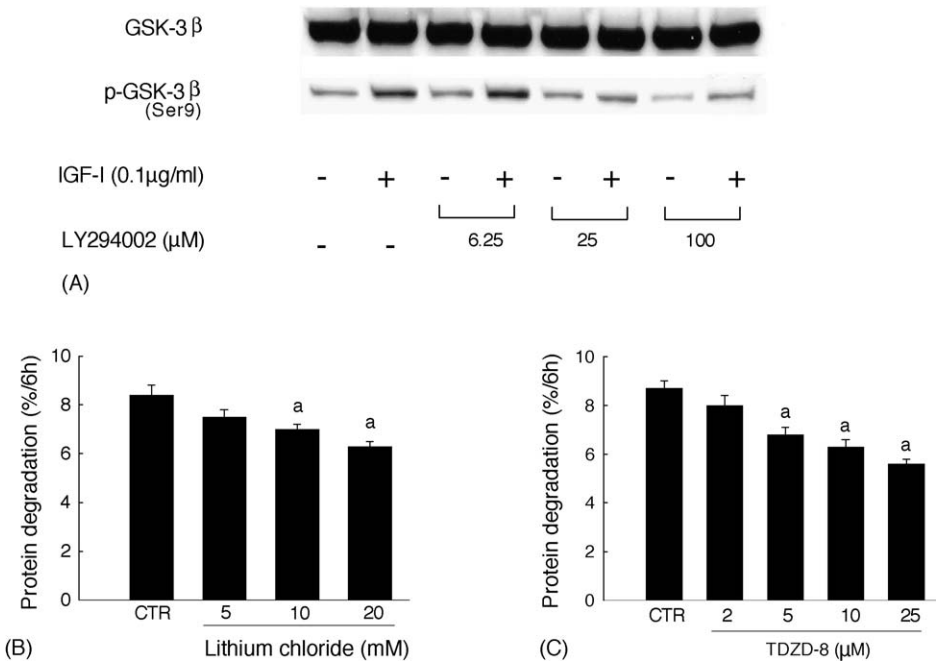

Fig. 3. (A) The effects of IGF-I and LY294002 on the expression of total and phosphorylated GSK-3β in dexamethasone-treated myotubes. Myotubes were cultured for 6 h in the presence of 1 μM dexamethasone and in the absence or presence of 0.1 μg/ml IGF-I or LY294002 as indicated in the figure. (B) The effect of LiCl on protein degradation in dexamethasone-treated myotubes. All myotubes were cultured in the presence of 1 μM dexamethasone. Control myotubes (CTR) were cultured in the presence of 20 mM NaCl. (C) The effect of TDZD-8 on protein degradation in dexamethasone-treated myotubes. All myotubes were cultured for 6 h in the presence of 1 μM dexamethasone. Control myotubes (CTR) were treated with the same concentration of DMSO that was used for addition of TDZD-8 to the culture medium. Results are means ± S.E.M. with $n = 6$ in each group. (a) $p < 0.05$ vs. CTR.

GSK-3β activity independent of phosphorylation as was also reported elsewhere (Martinez et al., 2002).

3.4. Inhibition of mTOR suppresses the anti-catabolic effects of IGF-I in dexamethasone-treated myotubes

In addition to GSK-3β, mTOR is a downstream target of PI3K/Akt and activation of mTOR results in phosphorylation of $p70^{S6K}$ and 4E-BP1 (Ferrari & Thomas, 1994). In order to examine the potential role of mTOR in IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes, we next determined myotube levels of phosphorylated $p70^{S6K}$ and 4E-BP1. Treatment of the myotubes with IGF-I resulted in increased levels of p-$p70^{S6K}$ and p-4E-BP1, consistent with mTOR activation (Fig. 5A). This was further confirmed by treatment of the myotubes with the mTOR inhibitor rapamycin that resulted in

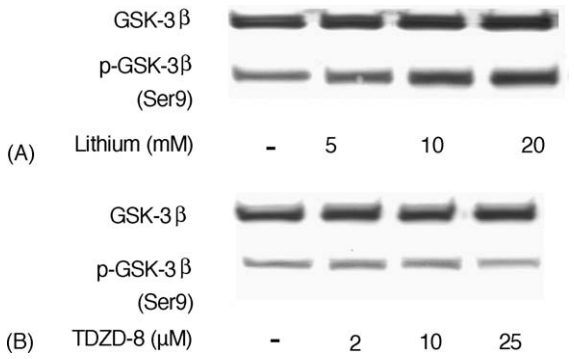

Fig. 4. The effects of LiCl (A) and TDZD-8 (B) on the expression of total and phosphorylated GSK-3β in dexamethasone-treated myotubes. Myotubes were cultured for 6 h in the presence of 1 μM dexamethasone and in the absence or presence of different concentrations of LiCl or TDZD-8.

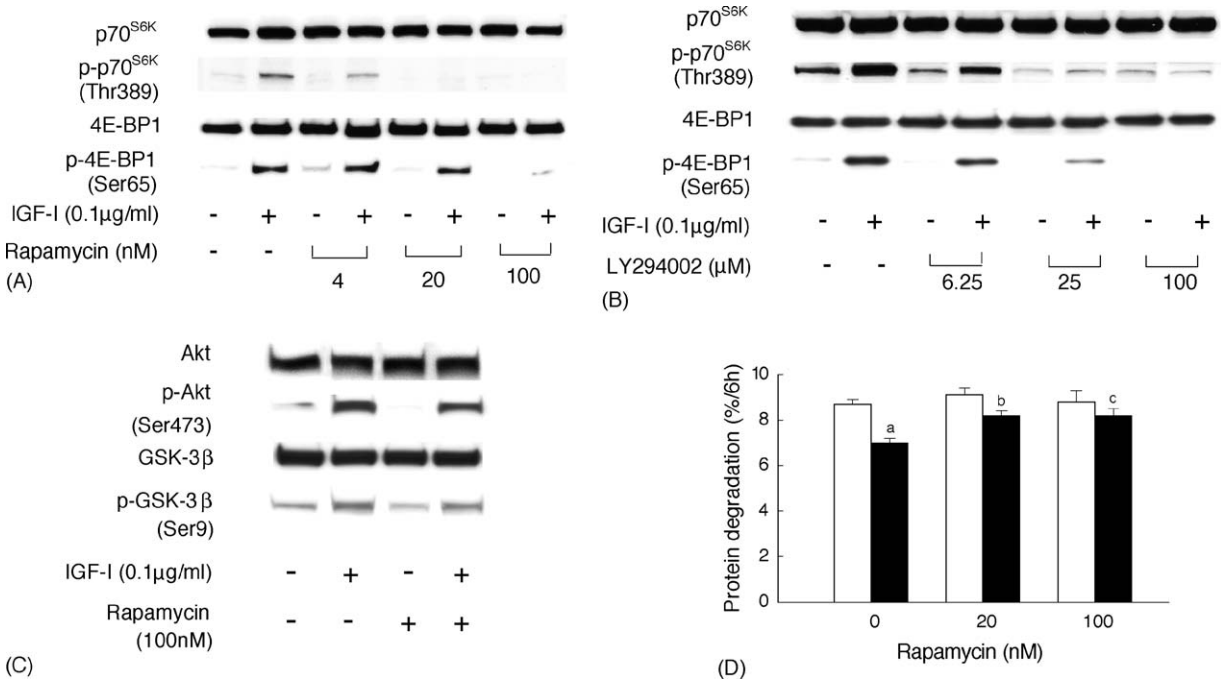

Fig. 5. (A) The effects of IGF-I and rapamycin on the expression of total and phosphorylated p70^{S6K} and 4E-BP1 in dexamethasone-treated L6 myotubes. (B) The effects of IGF-I and LY294002 on the expression of total and phosphorylated p70^{S6K} and 4E-BP1 in dexamethasone-treated myotubes. (C) The effects of IGF-I and rapamycin on the expression of total and phosphorylated Akt and GSK-3β in dexamethasone-treated myotubes. (D) The effect of IGF-I (filled bars) on protein degradation in dexamethasone-treated myotubes cultured in the presence of different concentrations of rapamycin as indicated in the figure. Results are means ± S.E.M. with $n = 6$ in each group. (a and b)$p < 0.05$ vs. corresponding no IGF-I (open bars); (a) $p < 0.05$ vs. (b and c).

a dose-dependent inhibition of IGF-I-induced phosphorylation of p70^{S6K} and 4E-BP1 (Fig. 5A). Treatment of the myotubes with LY294002 also blocked the IGF-I-induced phosphorylation of p70^{S6K} and 4E-BP1 suggesting that mTOR activation was downstream of PI3K/Akt under the present experimental conditions (Fig. 5B). This was an important observation because mTOR can be activated by PI3K/Akt-independent mechanisms as well (Patti, Brambilla, Luzi, Landaker, & Kahn, 1998). Rapamycin did not influence IGF-I-induced phosphorylation of Akt or GSK-3β (Fig. 5C), consistent with the concept that GSK-3β and mTOR are separate signaling pathways downstream of PI3K/Akt.

We next examined the influence of rapamycin on protein degradation in dexamethasone-treated myotubes. Rapamycin suppressed the IGF-I-induced inhibition of protein degradation in a dose-dependent fashion (Fig. 5C). The inhibition by IGF-I of protein degradation in dexamethasone-treated myotubes was approximately 20, 10, and 7% in the presence of 0, 20, and 100 nM rapamycin, respectively. Taken together, the results in Fig. 5 suggest that the IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes may at least in part reflect activation of mTOR.

3.5. Inhibition of MAP kinase signaling does not prevent the inhibitory effect of IGF-I on protein degradation in dexamethasone-treated myotubes

In addition to PI3K/Akt signaling, there is evidence that IGF-I can activate MAP kinase signaling as well (Tsakiridis et al., 2001). In order to test whether this mechanism may be involved in the IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes, we next measured myotube levels of phosphorylated Erk1/2 (p-Erk1/2) and p38 (p-p38). Treatment of the myotubes with IGF-I resulted in

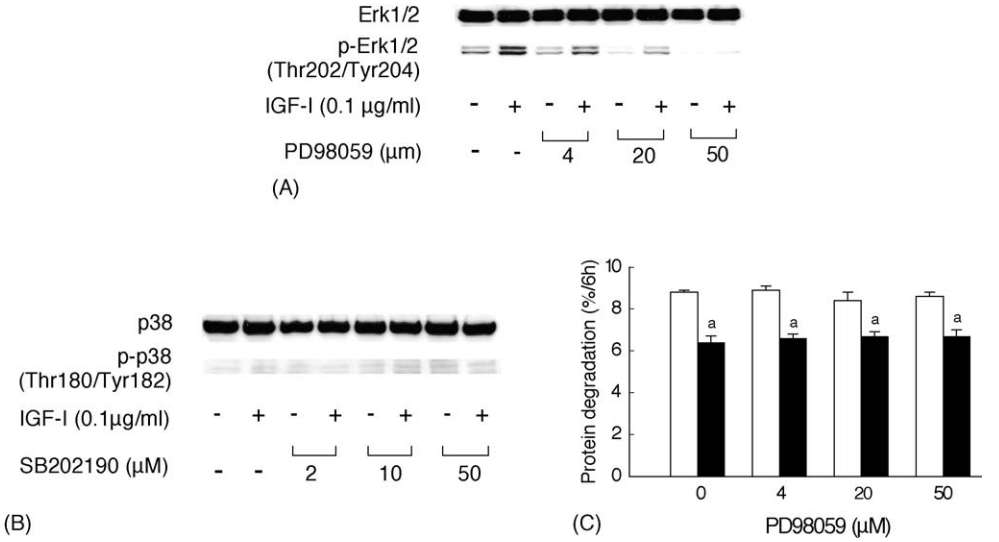

Fig. 6. (A) The effects of IGF-I and PD98059 on the expression of total and phosphorylated Erk1/2 in dexamethasone-treated myotubes. (B) The effects of IGF-I and SB202190 on the expression of total and phosphorylated p38 in dexamethasone-treated myotubes. (C) The effect of IGF-I (filled bars) on protein degradation in dexamethasone-treated myotubes cultured in the presence of different concentrations of PD98059 as indicated in the figure. Results are means ± S.E.M. with $n = 6$ in each group. (a) $p < 0.05$ vs. no IGF-I (open bars).

increased levels of p-Erk1/2, consistent with activation of this pathway (Fig. 6A), but did not influence p-p38 levels (Fig. 6B). Treatment of the myotubes with the Erk1/2 inhibitor PD98059 resulted in a dose-dependent reduction of p-Erk1/2 levels with an almost complete abolishment of p-Erk1/2 levels at the highest concentration (50 μM) of PD98059 (Fig. 6A). Interestingly, PD98059 did not influence the IGF-I-induced inhibition of protein degradation, not even at the highest concentration tested (Fig. 6C). This result strongly suggests that Erk1/2 was not involved in the IGF-I-induced inhibition of protein degradation under the present experimental conditions. Similarly, treatment of the myotubes with the p38 inhibitor SB202190 did not influence the IGF-I-induced inhibition of protein degradation (data not shown) or p-p38

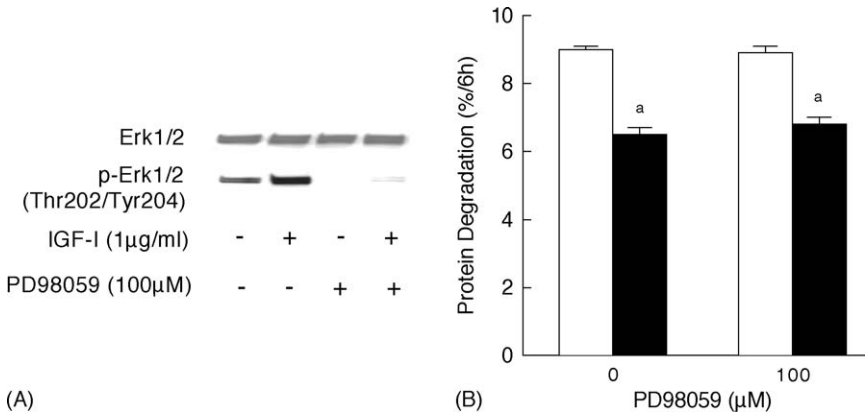

Fig. 7. The effects of 1 μg/ml of IGF-I and 100 μM PD98059 on (A) the expression of total and phopshorylated Erk1/2 and (B) protein degradation in dexamethasone-treated myotubes. Myotubes were cultured in the absence (open bars) or presence (filled bars) of 1 μg/ml of IGF-I. Results are means ± S.E.M. with $n = 6$ in each group. (a) $p < 0.05$ vs. no IGF-I.

levels in dexamethasone-treated myotubes (Fig. 6B). We next tested whether a higher dose of IGF-I would result in Erk1/2-dependent inhibition of protein degradation. Treatment of the myotubes with 1 μg/ml of IGF-I increased the levels of p-Erk1/2 and this effect of IGF-I was completely abolished by 100 μM PD98059 (Fig. 7A). The inhibition of protein degradation caused by 1 μg/ml of IGF-I, however, was not affected by 100 μM PD98059 (Fig. 7B). These results further support the concept that Erk1/2 MAP kinase signaling is not involved in the IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes, not even at a high concentration of IGF-I.

4. Discussion

In the present study, we examined potential mechanisms involved in the IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes. This experimental model was used because glucocorticoids are an important mediator of muscle wasting (Hasselgren, 1999) and treatment of cultured myotubes with dexamethasone results in increased ubiquitin-proteasome-dependent proteolysis, similar to the situation seen in skeletal muscle after burn injury and during sepsis (Wang et al., 1998). Results observed here suggest that IGF-I-induced inhibition of protein degradation at least in part reflects PI3K/Akt-mediated inactivation of GSK-3β and activation of mTOR. In contrast, the MAPK signaling pathway does not seem to be involved in IGF-I-induced inhibition of myotube protein degradation, despite the fact that Erk1/2 was activated by IGF-I.

The mechanisms of IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes were examined in the present study because previous reports suggest that proteolysis in certain muscle-wasting conditions can be inhibited by IGF-I (Fang et al., 1997, 1998, 2002; Rommel et al., 2001; Sacheck et al., 2004). The importance of IGF-I in the context of muscle wasting is further illustrated by the fact that the levels of circulating IGF-I and muscle IGF-I mRNA concentrations are reduced in catabolic conditions, including thermal injury, and that administration of IGF-I in those conditions reverse the catabolic response (Abribat, Brazau, Davignon, & Garriel, 1993; Lang, Liu, Nystrom, & Frost, 2000; Lang, Nystrom, & Frost, 2002). Circulating levels of IGF-I, most of which is probably produced by the liver, are in the range of 0.2–1 μg/ml in humans and rats (Humbel, 1990; Lang et al., 2000, 2002; Strock, Singh, Abdullah, Miller, & Herndon, 1990). The concentrations of IGF-I used in the present experiments (0.1 and 1 μg/ml), therefore, are within the normal range of circulating IGF-I levels. Local levels of IGF-I are lower in most, if not all, tissues and are attributed to a great part to local production of the hormone (Humbel, 1990). Although IGF-I at high concentrations may interact with the insulin receptor, there is evidence that IGF-I at concentrations below 50 nM (approximately 0.375 μg/ml) has only minimal interaction with the insulin receptor (Rubin, Shia, & Pilch, 1983). Thus, the IGF-I concentration used in our experiments (0.1 μg/ml) most likely did not result in insulin receptor binding.

The present results are in line with a recent report by Rommel et al. (2001) in which evidence was found that the PI3K/Akt/GSK-3β and PI3K/Akt/mTOR pathways mediated the hypertrophic effects of IGF-I in myotubes, although in those experiments the influence of IGF-I on protein degradation was not examined. In the same study, transfection of muscle cells with a dominant negative GSK-3β plasmid resulted in myotube hypertrophy, supporting results from the present experiments in which pharmacological inhibition of GSK-3β with LiCl or TDZD-8 resulted in reduced protein degradation. Additional support for a role of GSK-3β in the regulation of muscle mass was found by Vyas et al. (2002) who reported that GSK-3β negatively regulates skeletal muscle hypertrophy. In another study, Bodine et al. (2001) found evidence that mTOR signaling prevented muscle atrophy caused by denervation in vivo. The present results extend the previous observations (Rommel et al., 2001; Bodine et al., 2001; Vyas, Spangenburg, Abraham, Childs, & Booth, 2002) by determining that the PI3K/Akt/GSK-3β and PI3K/Akt/mTOR pathways regulate the inhibitory effect of IGF-I on protein degradation. This is important because there is evidence that muscle wasting in various catabolic conditions is mainly caused by increased protein breakdown and mechanisms reducing protein degradation may be particularly important for the prevention and treatment of muscle wasting.

In recent experiments, we found evidence that IGF-I inhibited protein breakdown in muscle from burned rats by a PI3K/Akt/GSK-3β-dependent mechanism

whereas mTOR activation was not involved (unpublished observations). Thus, some, but not all, mechanisms involved in IGF-I-induced inhibition of protein degradation may be similar when muscle atrophy is caused by different stimuli.

It should be noted that the conclusion that GSK-3β inactivation and mTOR activation were involved in the anti-catabolic effects of IGF-I was made from experiments in which different inhibitors were used. The potential non-specific effects of the various drugs used here need to be considered when the results are interpreted. Although LiCl has been shown in several studies to inhibit GSK-3β activity (Ryves & Harwood, 2001; Vyas et al., 2002), the compound may act as an inhibitor of other kinases as well (Davies, Reddy, Caivano, & Cohen, 2000). TDZD-8 has been reported to be a specific GSK-3β inhibitor at concentrations used in the present experiments and non-specific inhibition of other kinases was seen only at 10-fold higher concentrations (Martinez et al., 2002). Rapamycin is usually considered a specific mTOR inhibitor, exerting its effect by forming a complex with mTOR (Schmelzle & Hall, 2000). The inhibition of $p70^{S6K}$ and 4E-BP1 phosphorylation and the lack of effect on Akt and GSK-3β phosphorylation by rapamycin observed in the present experiments support the specificity of the drug as an mTOR inhibitor.

In addition to phosphorylation of GSK-3β and mTOR, phosphorylation (inactivation) of Foxo transcription factors is an important downstream mechanism of PI3K/Akt-mediated metabolic effects of IGF-I (Sandri et al., 2004; Stitt et al., 2004). In those experiments, inactivation of Foxo transcription factors by IGF-I resulted in reduced expression of the ubiquitin ligases atrogin-1 and MuRF1. It should be noted that the present results do not necessarily contradict previous reports on the role of Foxo transcription factors but instead offer evidence supporting the concept that multiple mechanisms downstream of PI3K/Akt may be involved in the effects of IGF-I in skeletal muscle (Fig. 8). The potential cross-talk between the different pathways downstream of PI3K/Akt also needs to be kept in mind for a complete understanding of mechanisms regulating the effects of IGF-I in skeletal muscle.

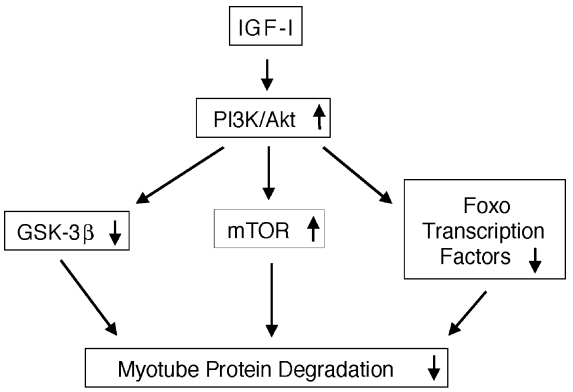

Fig. 8. Diagram illustrating different signaling pathways that are involved in IGF-I-induced inhibition of protein degradation in dexamethasone-treated myotubes. The present study provides evidence for a role of GSK-3β inactivation and mTOR activation. Evidence for a role of inhibited activity of Foxo transcription factors was reported elsewhere (Sandri et al., 2004; Stitt et al., 2004). Note that the upward and downward arrows in the figure refer to increased and decreased activity, respectively. The activity of PI3K/Akt and mTOR is increased by phosphorylation whereas the activity of GSK-3β and Foxo transcription factors is decreased by phosphorylation.

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