IGF-I and insulin receptor signal transduction in trout muscle cells

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ABSTRACT

In this study, primary cultures of trout skeletal muscle cells were used to investigate the main signal transduction pathways of insulin and IGF-I receptors in rainbow trout muscle. At different stages of *in vitro* development (myoblasts on day 1, myocytes on day 4 and fully developed myotubes on day 11), we detected in these cells the presence of immunoreactivity against ERK 1/2 MAPK and Akt/PKB proteins, components of the MAPK and the PI3K-Akt pathways, respectively, two of the main intracellular transduction pathways for insulin and IGF-I receptors. Both insulin and IGF-I activated both pathways, although the latter provoked higher immunoreactivity of phosphorylated MAPKs and Akt proteins. At every stage, increases in total MAPK immunoreactivity levels were observed when cells were stimulated with IGF-I or insulin, while total Akt immunoreactivity levels changed little under stimulation of peptides. Total Akt and total MAPK levels increased as skeletal muscle cells differentiated in culture. Moreover, when cells were incubated with IGF-I or insulin, MAPK-P immunoreactivity levels showed greater increases over the basal levels on days 1 and 4, with no effect observed on day 11. Although Akt-P immunoreactivity displayed improved responses on days 1 and 4 as well, a stimulatory effect was still observed on day 11. In addition, the present study demonstrates that purified trout insulin receptors possess higher phosphorylative activity per unit of receptor than IGF-I receptors. In conclusion, these results indicate that trout skeletal muscle culture is a suitable model to study the insulin and IGF-I signal
transduction molecules and that there is a different regulation of MAPK and Akt pathways depending on the developmental stage of the muscle cells.

INTRODUCTION

The actions of insulin and IGF-I on fish skeletal muscle have been widely studied. Specifically, IGF-I effects on growth are well characterized (Gray and Kelley, 1991; McCormick et al., 1992; Kelley et al., 1993; Duan et al., 1995), as are their role in stimulating both differentiation (Greene and Chen, 1997; Perrot et al., 1999) and reproduction (Maestro et al., 1997). Moreover, recent data suggests a role for IGF-I in fish skeletal muscle metabolism (Castillo et al., 2004). Insulin is a hypoglycemic hormone, and muscle constitutes its principal target in fish. Therein, insulin stimulates glucose transport, amino acid uptake, and accelerates muscle protein synthesis. In addition, insulin plays a role as a general mitogen and growth promoter (reviewed by Mommsen, 2001)

These various stimulatory actions of IGF-I and insulin are ultimately dependent upon peptides binding to their respective cell surface receptors. Both receptors are heterotetrameric, consisting of two extra cellular α subunits and two β subunits crossing the cell membrane, which possesses a tyrosine kinase activity domain within its intracellular area (Le Roith et al., 1995a, Stewart and Rothwein, 1996). In mammalian muscle, insulin receptors predominate over IGF-I receptors, while fish skeletal muscle exhibits higher levels of IGF-I receptors than insulin receptors (Párrizas et al., 1995 a, b). Based on these findings, we have recently shown the abundance of IGF-I receptors in trout skeletal muscle myosatellite cells
during periods of low insulin binding (Castillo et al., 2002). Taken together, these data suggested an important role for IGF-I in fish skeletal muscle. This was confirmed more recently when IGF-I was discovered to stimulate glucose and alanine uptake in trout muscle cells to a greater extent than insulin. Furthermore, IGF-I was found to stimulate cell proliferation in this model while insulin did not (Castillo et al., 2004).

Different studies have examined insulin and IGF-I receptor tyrosine kinase activity (TKA) in both mammalian (James et al., 1986; Zorzano et al., 1988) and fish skeletal muscle (Párrizas et al., 1995b). Indeed, the authors addressed the entire fish life cycle (Méndez et al., 2001), and showed that IGF-I receptors exhibit higher TKA than insulin receptors, a result that is contrary to that described in mammalian (where insulin receptors have higher TKA than IGF-I receptors); moreover, fish muscle exhibits lower TKA levels than mammalian muscle (Párrizas et al., 1995b). These differences are undoubtedly related to the potentially different roles played by insulin and IGF-I in fish skeletal muscle (Párrizas et al., 1995b; Planas et al., 2000b; Castillo et al., 2004).

Cross-talk between these growth-related hormones is widespread: IGF-I and insulin converge via common message transduction systems, including the PI3 kinase and MAPK cascades, while IGF-I and insulin can potentially bind to each other’s receptors. Mammalian studies have shown that following receptor auto-phosphorylation in tyrosine residues, the signal is transmitted downstream (White and Kahn, 1993), thereby phosphorylating other cell proteins in turn. (Reviewed in Myers and White, 1993; and Schaeper et al., 2000). For skeletal muscle insulin and IGF-I receptors, this signal may be transmitted by two main methods: the PI3K-Akt
and MAPK pathways. IGF-I is able to stimulate both proliferation and differentiation of muscle cells in culture (Ewton et al., 1994; Rosenthal and Cheng, 1995; Engert et al., 1996; Florini et al., 1996). Proliferation as a response to IGF-IR signaling is mainly mediated through the activation of the MAPK pathway, which has been implicated in increasing muscle cell proliferation (Coolican et al., 1997; Day et al., 1999; Lawlor et al., 2000; Jones et al., 2001; Leshem et al., 2002), whereas signaling through the PI3K-Akt pathway mediates such crucial effects in skeletal muscle as myoblast differentiation and anabolic effects, including glucose and amino acid uptake (Florini et al., 1996; Coolican et al., 1997; Calera et al., 1998; Kaliman et al., 1998). Active Akt induces mammalian myotube hypertrophy (Rommel et al., 1999), while in myotubes, but not in myoblasts, Akt is able to inhibit MAPK activation (Rommel et al., 1999; Zimmermann et al., 1999).

The MAPK and PI3K-Akt pathways exert completely different effects in mammalian skeletal muscle, and the mechanism by which myoblasts switch from a proliferative to a differentiative signal still remains unclear (review by LeRoith, 2000).

In fish, very little is known about the IGF-I and insulin signaling pathways. Although Pozios et al., (2001) showed that IGF-I stimulates proliferation of zebrafish embryonic cells by activating the MAPK and PI3-kinase signaling pathways, no studies on IGF-I or insulin intracellular signaling in fish skeletal muscle have been performed thus far, mainly due to the lack of an appropriate in vitro model of fish muscle growth and development. In this respect, the development of a technique for the primary culture of rainbow trout skeletal muscle cells (Rescan et al., 1995a; Fauconneau and Paboeuf, 2000) has opened up possibilities for studies on insulin
and IGF-I actions *in vitro*, as we have previously conducted (Castillo et al., 2002; Castillo et al., 2004).

The present study, which employed a chromatographic technique to separate insulin and IGF-I receptors, as well as a primary culture of trout skeletal muscle cells, comprised several aims: 1) to compare the intrinsic tyrosine kinase activity of the insulin and IGF-I receptors in fish; 2) to identify components of the intracellular signaling cascades of insulin and IGF-I in trout skeletal muscle cells; and 3) to determine their activation in response to both peptides throughout the *in vitro* differentiation of skeletal muscle cells.

MATERIALS AND METHODS

***Chemicals***

Wheat germ agglutinin (WGA), bound to agarose, was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Affi-Gel 10 (cat. nº 153-6099) was bought from Bio-Rad Laboratories (Hercules, California, USA). Recombinant porcine insulin was obtained from Lilly Co. (Indianapolis, IN, USA), human recombinant IGF-I from Peninsula Laboratories, Inc. Europe Ltd. (Merseyside, UK), and trout recombinant IGF-I from GroPep (Adelaide, Australia). Recombinant human Tyr A14-[125I]-insulin, 3-[125I] IGF-I, with a specific activity of 2000 Ci mmol⁻¹, and [32P]-ATP, with a specific activity of 5000 Ci mmol⁻¹, were purchased from Amersham Pharmacia Biotech Europe GmbH (Barcelona, Spain). Anti-phospho-p44/42 (cat nº
9106), anti-Akt (cat nº 9272), anti-Akt-P (cat nº 9271) and anti-p44/42 antibodies (cat nº 4696) were ordered from Cell Signaling Technology Inc. (Beberly, MA, USA). Anti-PI3K (against the SH2 domain of the N-terminal region, cat nº 06-195) was bought from Upstate Biotechnology (Lake Placid, NY, USA). Reactives for quimioluminiscence (ECL) were obtained from Amersham Life Sciences (Little Chalfont, Buckinghamshire, England) and reactives for electrophoresis from Bio-Rad Laboratories (Hercules, California, USA). Disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, IL, USA) and the remaining reactives were purchased from Sigma-Aldrich Química, S.A (Madrid, Spain).

Animals and cell cultures

Rainbow trout (*Oncorhynchus mykiss*) experimental animals were obtained from the Piscifactoria Truites del Segre (Oliana, Lleida) and maintained in the Servei d’Estabulari of the Faculty of Biology, University of Barcelona, as described previously (Castillo et al., 2002). For IGF-I and insulin receptor signal transduction experiments, primary cultures of rainbow trout skeletal muscle cells were carried out: for each culture, 30 to 60 animals, each weighting approximately 5 g, were killed by a blow to the head and then immersed for 30 seconds in 70% ethanol to sterilize external surfaces. Cells were isolated, pooled, and cultured following previously described protocols (Rescan et al., 1995a; Castillo et al., 2004). All experiments were conducted with cells seeded at a density of 1.5 to 2 x 10⁶ per well, in six-well plastic plates (9.6 cm²/well, NUNC). Observations of morphology were conducted regularly to control the cell state, which were used on days 1 (mononucleated cells), 4 (mostly small myotubes) and 11 (big myotubes). All
experiments were performed in triplicate, as was each condition (3 wells). Cells were incubated at 18°C, the optimal temperature for culture growth.

For IGF-I and insulin receptor experiments, brown trout larvae (*Salmo trutta*) obtained from the Piscifactoria de Bagà (Barcelona, Spain) were used. Samples were frozen directly in liquid nitrogen, where they were kept until the insulin and IGF-I receptors were purified.

**Separation of IGF-I and insulin receptors**

The first step in separating insulin and IGF-I receptors by differential centrifugation was conducted in *Salmo trutta* larvae following the protocol described by Maestro et al., (1998). Frozen samples (7g) were homogenized in a Tris-HCl buffer (25 mM Tris-HCl, 5 mM CaCl₂, pH 7.6) and centrifuged at 600 × g for 10 minutes at 4°C. Supernatant was taken and centrifuged again at 40,000 × g for 30 minutes at 4°C. Both pellets were resuspended in a buffer containing 25 mM HEPES, 4 mM EDTA, 4 mM EGTA, 2 mM PMSF, 1 mM bacitracin, 1 mM leupeptin, 1 mM pepstatin, and 25 mM benzamidine, pH 7.6. Membrane solubilization was performed by adding Triton X-100 to a final concentration of 2%, which was maintained in agitation for 1h at 4°C. Subsequently, the sample was centrifuged at 150,000 × g for 90 minutes at 4°C, and supernatant was passed three times through a WGA-agarose column. After washing the column with buffer (25 mM HEPES and 0.1% Triton X-100, pH 7.6), receptors were eluted with washing buffer supplemented by N-acetyl-D-glucosamine 0.3N.

Both the insulin and IGF-I receptors were purified following the methods described by Yu et al., (1986) and Tollefsen et al., (1987) with certain modifications. This technique is based on the coupling of a cold peptide (insulin or IGF-I) to an
agarose gel (Affigel 10, Bio-Rad Laboratories, S.A, Madrid), thereby obtaining either an insulin-agarose or an IGF-I-agarose column. Briefly, to obtain the latter, agarose gel was incubated with 30 µg of IGF-I in a coupling buffer (0.1 M Hepes, 6 M urea, 80 M CaCl₂, pH 7.6) for 4 h at 4ºC. For insulin-agarose, incubation was performed using 45 µg of cold insulin, and under the same time and temperature conditions. The coupling reaction was halted by adding ethanolamine 1M at pH 8.0. The resulting columns were equilibrated with C buffer (0.1 M Hepes, 6M urea, pH 7.6) and were then ready for use. To separate IGF-IR, samples (Salmo trutta larvae) semi-purified in a WGA-agarose column were diluted with C buffer and incubated with 1µl of insulin 350 µM to saturate the INS-R for 30 min at 22ºC (this step was not necessary for insulin-agarose). Afterwards, the sample was saturated with a solution of IGF-I-agarose (in a 12:1 proportion, sample: IGF-I-agarose) and the entire complex was incubated for 16 hours at 4ºC with agitation. The mixture was then passed through a column, where the IGF-I bound to the agarose-fixed IGF-I receptors. After eluting the sample with buffer (10 mM sodium acetate, 1.5 M NaCl, 0.1 % Triton X-100, 10 % glycerol, pH 5.0) and neutralizing it with Tris-HCl pH 7.4, several fractions containing purified IGF-IR were obtained. By substituting cold IGF-I with cold insulin, the steps followed for INS-R purification were identical.

**Western blotting**

To determine the intracellular signal transduction pathways activated by insulin and IGF-I during *in vitro* development of skeletal muscle cells in culture,
Western blots against several proteins involved in the PI3K-Akt and MAPK pathways were performed.

In these experiments, 30-50 fish, rainbow trout (*Oncorhynchus mykiss*) each weighting approximately 5 g, were used for every culture. After pooling cells from all animals of the same culture, the cells were seeded at a density of 1.5 to 2 x 10^6 per well in six-well plastic plates. Following 1, 4 or 11 days in culture, the cells were incubated with DMEM + 0.5% BSA for 2-3 hours. Afterwards, they were incubated with DMEM+ 0.5% BSA and a fixed concentration of peptides (100 nM for IGF-I or 1 µM for insulin) for 30 minutes. Subsequently, the medium was aspirated, the wells were washed with ice-cold PBS, and the cells were lysed with lysis buffer (1 % NP-40, 0.4 mM sodium orthovanadate, 10 mM Tris, 140 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, pH 7.6). After obtaining cell lysates, protein content was determined with the Bradford reagent method (Bradford, 1976) and electrophoresis using a polyacrilamide gel was conducted in the presence of SDS (SDS-PAGE) (each lane was loaded with 50 µg of protein). Samples were then transferred to a PVDF membrane for 90 minutes under a constant current of 1A in a transfer buffer (25 mM Tris-HCl, 190 mM Glycine, 20% Methanol, pH 7.5). Following a 30-minute wash (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.5), the membrane was incubated overnight at 4°C with the primary antibody (diluted in a washing buffer, at the dilution indicated at the bottom of each figure). The primary antibodies detected the presence of mitogenesis activator protein kinase (MAPK) and its active form (MAPK-P), as well as the Akt protein and its phosphorylated form (Akt-P).
The membrane was then washed for 30 minutes and incubated for 1 hour at room temperature with the corresponding secondary antibody at the indicated dilution. Immuno-reactive bands were visualized by ECL and quantified with an image analyzer (TotalLab v1.00, Nonlinear Dynamics Ltd, 2000).

To better characterize the signal transduction pathways, we analyzed the effects of two inhibitors of the cell signalling: the PD-98059 is an inhibitor of the MEK1 protein, a component of the MAPK pathway, while wortmannin is an inhibitor of the PI3K-Akt pathway. Cells were preincubated for 30 min with wortmannin (1 µM) or PD-98059 (50 µM), and IGF-I was added for 30 additional minutes.

**Binding assays (in purified receptor preparations)**

Binding experiments were performed in purified receptor preparations from *Salmo trutta* larvae following the method described by James et al., (1986). A 50 µl eluted sample from an insulin-agarose column and a 40 µl eluted sample from an IGF-I-agarose column (5- to 10-fold concentrations by centrifugation at 5000 × g for 15 minutes at 4°C using Filtron 30K Microsep Centrifugal Concentrators), corresponding to approximately 3 µg of INS-R and 3 µg of IGF-IR, respectively (Bradford method, Bio-Rad Laboratories, S.A, Madrid), were incubated for 16h at 4°C in a buffer of 30 mM HEPES, including 0.1% BSA and 100 units/ml of bacitracin (pH 7.6), with increasing concentrations of non-labeled hormone (ranging from 0.0125 nM to 100 nM porcine insulin or recombinant human IGF-I) and the radioactive ligand in trace concentrations (25 pM). Receptors which bound to the ligand were precipitated with
bovine γ-globulin (0.08%) and polyetilenglicol (10.4% w/v), and then centrifuged at 14,000 × g for 7 min at 4°C. Non-specific binding was calculated as follows: a radioactive-bound ligand, in the presence of cold hormone at a concentration of 100 nM, was subtracted from the total count.

**Tyrosine kinase activity assays**

Tyrosine kinase activity (TKA) was determined in preparations of purified receptors from *Salmo trutta* larvae according to the method described by James et al., (1986). Purified receptors (3-4 µg of protein) were incubated for 16 h at 4°C with cold insulin or IGF-I (60 nM, final concentration) in an HEPES buffer in the presence of 100 mM MgCl₂, pH 7.4. Samples were later incubated with 50 µM [³²P]-ATP for 10 minutes to allow receptor auto-phosphorylation. A synthetic substrate poly (Glu:Tyr; 4:1) was added at a final concentration of 0.25 mg/ml and incubated for 30 min. The reaction was halted by transferring the samples to filter paper (Whatmann 3MM), washed in 10% tricloroacetic acid with 10 mM sodium pyrophosphate. Radioactivity was recorded on filter paper using a scintillation counter.

**Statistical analysis**

The treatments were performed in triplicate for each experiment, and each experiment was performed three times. Data are presented as mean ± standard error. For the receptor purification experiments, at a minimum, each binding experiment was performed in duplicate using receptors from different purifications. Statistical
differences between conditions were tested with one-way ANOVA, followed by the Tukey’s test. Results were considered statistically significant at $P < 0.05$

RESULTS

**TKA and binding in preparations of purified IGF-I and insulin receptors**

We studied the stimulation of tyrosine kinase activity (TKA) by IGF-I and insulin in receptor preparations from trout larvae (*Salmo trutta*), purified using an insulin-agarose column (INS-R purification) and an IGF-I-agarose column (IGF-IR purification).

In samples eluted from the insulin-agarose column (preparations containing a high proportion of INS-R), insulin stimulated phosphorylation of the exogenous substrate 122.43% above the basal level. In preparations obtained from the IGF-I-agarose column (containing a high percentage of IGF-IR), IGF-I stimulated TKA 162.98% above the basal level (Table 1). In this table we also record the binding observed in these preparations for each peptide and the ratio of TKA/ binding, where insulin receptors exhibited a 3-fold greater activity than IGF-I receptors.

The cross-reaction of peptides both in the insulin-agarose and IGF-I-agarose columns resulted in binding values below 0.5% SpB/ 20 µg of protein, and therefore were discarded.

**Insulin and IGF-I signaling pathways in skeletal muscle**

To study the activation of different IGF-I and insulin receptor signal transduction pathways, we determined the presence of both total and phosphorylated
MAPK and Akt proteins throughout the different developmental stages of the trout skeletal muscle cells in a primary culture.

*MAPK pathway*

These Western blots were performed from lysates of rainbow trout skeletal muscle cells in primary cultures at different stages of development: muscle cells on day 1 of culture development, myoblasts (small myotubes formed by fusion of few cells) on day 4, and large myotubes on day 11. In Figure 1 we show a representative experiment as well as a densitometric analysis of the results: throughout development, from day 1 to day 11, total MAPK levels increased in control cells (Fig 1A). In addition, both IGF-I and insulin caused a significant increase in total MAPK levels when compared to the control sample on day 1 (non-differentiated cells), whereas on day 4 (small myotubes) and in fully differentiated myotubes (day 11) the insulin effects were not significant (Fig 1B). When treating myoblasts with the protein synthesis inhibitor cycloheximide, the effect on MAPK total levels caused by IGF-I was not observed (data not shown).

Although MAPK-P levels did not change significantly throughout development in control cells (data not shown), treatment with peptides did stimulate it, particularly on days 1 and 4. For this reason, the data presented in Figure 2 only displays the effects of peptides at each stage of development, normalized with the total MAPK content (MAPK-P/ MAPK). In myoblasts, IGF-I caused an almost 5-fold increase in MAPK-P levels on day 1 (440% above the control levels), whereas stimulation by insulin was slightly lower (435%). Both peptides also increased MAPK-P levels on day 4 (530% for IGF-I and 310% for insulin), whereas in
differentiated myotubes (day 11), the response was clearly lower than in previous stages, and there was no effect of insulin or IGF-I.

Table 2 shows the effect of PD-98059 (inhibitor of the MAPK signaling pathway) reducing the effects of IGF-I on MAPK phosphorylation (from 171% to 39 above basal values).

**PI3K-Akt pathway**

For each stage of development, no differences in Akt levels were observed between the peptide-stimulated and control cells (data not shown). Additionally, throughout development, from day 1 to 11, there were increases in total Akt protein levels. The highest levels, presented in fully differentiated myotubes, occurred on day 11 in basal- and peptide-stimulated cells (Figure 3).

Figure 4 reveals that on day 1, IGF-I caused an approximate 6.5-fold increase in Akt-P levels (normalized with total Akt) (645% over the basal levels), although there was not a significant change between the effect on Akt-P at day 1 and day 4 (665% over basal levels). On the other hand, there was no effect of insulin at day 1 (362% over basal levels) whereas it significantly stimulated Akt-P at day 4 (520% over basal levels). Although on day 11 stimulation decreased compared to day 1 and day 4, both peptides still caused a significant increase in Akt-P levels, higher for IGF-I than insulin (323% vs. 230%, respectively). Preincubation of cells with wortmannin (inhibitor of the Akt signaling pathway) reduced the phosphorylation of IGF-I–stimulated Akt protein (from 396 % to 89 % above basal values) (Table 2)
DISCUSSION

This is the first study to describe and compare the relative phosphorylative capacity of both insulin and IGF-I receptors and the presence and activation of certain intracellular signaling pathway components in a fish species. Moreover, to the authors’ knowledge, this marks the first time that this kind of signaling pathway study has been conducted in a primary muscle culture in any fish species. Our results suggest that the insulin receptor possesses a higher phosphorylative capacity than the IGF-I receptor. In addition, we also demonstrate activation of the MAPK pathway during trout skeletal muscle cell proliferation, as well as activation of the PI3K-Akt pathway during differentiation and maturation.

**TKA of IGF-I and insulin receptors**

Here we describe the phosphorylation capacity of insulin and IGF-I receptors in trout, and therefore, their potential to develop intracellular signaling. To study TKA in IGF-I and insulin purified receptors, we used brown trout larvae, since our group described the stages of brown trout development where the binding of these peptides is highest (Méndez et al., 2001). Our aim was to compare the intrinsic TKA of both receptors to establish which of them possesses the greater phosphorylative capacity, thereby identifying the higher transmission potential of the intracellular signal and the resulting effects generated by that receptor. When comparing the TKA of these receptors we observed that while the IGF-I binding levels in the purified IGF-IR preparations were much higher (almost 4-fold) than the purified INS-R insulin preparations, IGF-I stimulation of TKA was only slightly higher,
although not significantly so than that for insulin (163 % vs. 122 %, respectively). In addition, when calculating the ratio between TKA and binding in the purified receptor preparations, we observed INS-R to have a ratio nearly 4 times greater than that for IGF-IR (24.73 vs. 7.31), indicating a higher phosphorylative capacity for INS-R compared to IGF-IR. These results confirm previous study hypotheses, since they are consistent with data obtained in several trout tissues (Leibush et al., 1996) as well as in other poikilotherms (Párrizas et al., 1995b). It has, moreover, been suggested that during evolution, INS-R seems to acquire a higher phosphorylative capacity in comparisons of mammals and fish (Planas et al., 2000).

Signal transduction in skeletal muscle

In mammals, the PI3K and MAPK pathways are the main signaling pathways for IGF-I and insulin (LeRoith et al., 1995; Butler et al., 1998). It has been postulated that such pathways exert opposite effects in muscle, with the respective consequences dependent on the myocyte differentiation state. Cross-talk can also exist, however, particularly at the Ras protein level, acting as a positive effector in both pathways (Katz and McCormick, 1997; Hunter, 1997).

The primary culture of trout skeletal muscle cells has been extensively shown as a very powerful in vitro tool to study the IGF-I and insulin role in fish skeletal muscle (Castillo et al., 2002; Castillo et al., 2004). Preliminary experiments identified the presence of these IGF-I and insulin signaling pathway components in these cells; PI3K, Akt, and MAPK, as well as their active forms, are all present in trout skeletal muscle cells, which can be observed when the cells are activated with either IGF-I or insulin (data not shown). To determine the activation of these
proteins throughout muscle development, cells at different stages of *in vitro* development were utilized, from myoblasts to fully differentiated myotubes. The incubation time for IGF-I or insulin in fish cells (30 minutes) has been shown to be sufficient for MAPK and Akt protein phosphorylation in a fish model, as described by Pozios et al., (2001) using zebrafish embryo cells. The specificity of the detected molecules was proved by the use of specific inhibitors of the MAPK and PI3K-Akt signaling pathways: when incubating trout skeletal muscle cells with the inhibitor wortmannin, we observed a decrease on Akt phosphorylation, as it has been previously described (Sakamoto et al., 2003; Shefer et al., 2003); on the other hand, the incubation of skeletal muscle cells with the inhibitor PD-98059 caused a decrease on the IGF-I–stimulated MAPK phosphorylation, in agreement with the results obtained by Pozios et al. (2001) on zebrafish cells.

In the cultured cells, we found that IGF-I and insulin caused an increase in total MAPK levels, particularly on day 1 (myoblasts), and that this effect decreased as the culture developed into myotubes by day 11. Experiments with the protein synthesis inhibitor cycloheximide (where a preincubation of cells with the inhibitor provoked a reduction of the IGF-I-stimulated increase of total MAPK levels) suggest that this increase of MAPK immunoreactivity levels could be linked to a synthesis of new protein, a hypothesis that should be confirmed in future studies. In addition, both IGF-I and insulin increased MAPK-P levels above the control in both myoblasts (day 1) and small myotubes (day 4), while there was no significant change in differentiated myotubes. Taken together, these results suggest that IGF-I and insulin activate the MAPK pathway in myoblasts but not in differentiated muscle cells. That is, activation of the MAPK pathway in trout skeletal muscle cells
is stage-dependent, an observation supported by previous studies in both mammalian (Samuel et al., 1999; Jones et al., 2001) and fish cells, where MAPK was activated by IGF-I-stimulated proliferation of embryonic cells (Pozios et al., 2001). IGF-I tended to provoke higher increase of both MAPK-P and Akt-P levels than insulin: this is not contradictory with the TKA/binding ratio of receptors, and it is explained by the higher number of IGF-I receptors of the trout skeletal muscle cells in culture (Castillo et al., 2002).

Total MAPK levels increased with differentiation of trout skeletal muscle cells, which may be linked to the reported increase in IGF-IR when these cells differentiate from myoblasts to myotubes (Castillo et al., 2002): a greater number of receptors in differentiated cells could give as a result increased basal levels of these proteins, and therefore the myogenic differentiation would yield a more IGF-I responsive phenotype. This is in agreement to Al-Khalili et al., (2004) who also described that increased total MAPK levels under basal conditions during human skeletal muscle cell differentiation could be related to a more insulin responsive state. The fact of the lower response of Akt-P and especially of MAPK-P to peptides in the differentiated myotubes at day 11 could be a consequence of the higher levels of control total Akt and total MAPK proteins, and as thus the activation of phosphorylation of both proteins by peptides could be lower.

Concerning the PI3K-Akt pathway, we found no alterations in total Akt levels in any of the studied stages when cells were incubated with insulin or IGF-I. However, as with MAPK, we did observe an increase in Akt levels throughout skeletal muscle cell development in culture. This increase in total Akt levels may be linked to the reported increase in IGF-I-stimulated glucose uptake levels when these
cells differentiate to myotubes in culture (Castillo et al., 2004). Calera and Pilch (1998) also reported a marked increase in endogenous Akt protein levels during differentiation of Sol8 skeletal muscle cells, and Gonzalez et al., (2004) described the increase of total Akt levels during differentiation of cultured mouse muscle cells. In addition, Altomare et al., (1998) observed increased Akt mRNA levels during C2C12 muscle cell differentiation and more recently Al-Khalili et al., (2004) described increased total Akt levels under basal conditions during human skeletal muscle cell differentiation. Insulin and IGF-I stimulated Akt phosphorylation, and such activation was observed throughout all developmental stages, from myoblasts to myotubes, with IGF-I tending to be more active than insulin, particularly in myoblasts, which suggests that the PI3k-Akt pathway is activated during all stages of trout skeletal muscle development. These results differ from data reported in other studies describing the PI3K-Akt pathway as more active in differentiated myotubes (Coolican et al., 1997; Rommel et al., 1999). Nevertheless, it has been reported that trout skeletal muscle cells in a primary culture can proliferate and differentiate at the same time, indeed from the earliest stages of in vitro development (Rescan et al., 1995a; Fauconneau and Paboeuf, 2000), a fact that may explain why insulin and IGF-I activate the Akt signaling pathway in trout myoblasts. After these initial steps, trout cells continue to differentiate, with little proliferation. This may explain the lack of significant change in MAPK-P levels detected in peptide-stimulated fully differentiated myotubes (day 11), although changes in Akt-P levels were significant.

In conclusion, the present study describes not only the differing activity of purified insulin and IGF-I receptors in trout, but also the IGF-I- and insulin-signaling pathways throughout the in vitro development of rainbow trout skeletal
muscle cells. The insulin receptor seems to be more active than IGF-IR in trout, since it exhibits higher tyrosine kinase activity per unit of receptor than IGF-IR. Moreover, IGF-I and insulin activate different intracellular signal transduction pathways in trout skeletal muscle cells, depending on the stage of cell culture development; in differentiating and proliferating myoblasts, both the MAPK and Akt pathways are activated by IGF-I and insulin, whereas in fully differentiated myotubes the peptides only activate the Akt pathway, although to a lower extent than in myoblasts.

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GRANTS

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Table 1 – Specific binding values and tyrosine kinase activity of the IGF-I and insulin purified receptor preparations. Results are expressed as mean ± standard error (n=3). Different letters indicate significant differences (P<0.05). SpB: Specific Binding; TKA: tyrosine kinase activity.

Fig. 1 – (A) MAPK levels in trout muscle cells during in vitro development. Cells were incubated with insulin (1µM) or IGF-I (100 nM) for 30 minutes, lysed, 50 µg of protein were loaded in each lane and subjected to 10% SDS-PAGE under reducing conditions. Primary antibody against MAPK (final dilution 1:200) and secondary antibody anti-mouse (1:2000) were used. Below this, densitometry analysis of three independent experiments is shown. (B) Effects of insulin and IGF-I treatment on MAPK levels at different stages of differentiation in culture. Different letters indicate significant differences (P<0.05).

Fig. 2– Stimulation of MAPK phosphorylation (MAPK-P) by insulin (1µM) and IGF-I (100 nM) on trout muscle cells at different developmental stages. Cells were incubated with peptides for 30 minutes, lysed, 50 µg of protein were loaded in each lane and subjected to a 10% SDS-PAGE under reducing conditions. Primary antibody against MAPK-P (final dilution 1:200) and secondary antibody anti-mouse (1:2000) were used. A densitometry analysis of three independent experiments,
normalized for the content of total MAPK, is shown below. Different letters indicate significant differences (P<0.05).

Fig. 3 – Akt levels in trout muscle cells during development. Cells were incubated with insulin (1µM) or IGF-I (100 nM) for 30 minutes, lysed, 50 µg of protein were loaded in each lane and subjected to 10% SDS-PAGE under reducing conditions. Western blot was performed with primary antibody against Akt (final dilution 1:200) and secondary antibody anti-rabbit (1:5000). A densitometry analysis of three independent experiments is shown below. Different letters indicate significant differences (P<0.05).

Fig. 4 – Stimulation of Akt phosphorylation (Akt-P) by insulin (1µM) and IGF-I (100 nM) in trout muscle cells at different stages of development. Cells were incubated with peptides for 30 minutes, lysed, 50 µg of protein were loaded in each lane and subjected to a 10% SDS-PAGE under reducing conditions. Primary anti Akt -P (final dilution 1:500) and secondary antibody against rabbit (1:5000) were used. Densitometry analysis of three independent experiments, normalized for the content of total Akt, is shown below. Different letters indicate significant differences (P<0.05).

Table 2 – Effect of inhibitors on the ERK 1/2 MAPK and PI3K- Akt pathways in IGF-I – stimulated myoblasts of trout. Cells were preincubated for 30 min with inhibitors, and IGF-I was added for 30 additional minutes. Results are expressed as percentage of stimulation over basal levels and mean ± standard error (n=3). Different letters indicate significant differences (P<0.05).
TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>TKA</th>
<th>%SpB/20µg protein</th>
<th>TKA / Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified INS-R</td>
<td>122.43±3.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.95±0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.73&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Purified IGF-IR</td>
<td>162.98±6.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.31±3.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Figure 1

CONTROL | INSULIN | IGF-I

MAPK

A

CONTROL | INSULIN | IGF-I

B

Day1 | Day4 | Day11

Day1 | Day4 | Day11

Day1 | Day4 | Day11

% stimulation (relative values)

% stimulation (relative values)

% stimulation (relative values)
Figure 2

Day 1 | Day 4 | Day 11
---|---|---
C | IGF-I | INS
---|---|---

Figure 3

CONTROL | INSULIN | IGF-I
---|---|---
Day 1 | Day 4 | Day 11
---|---|---

AKT

MAPK-P

% stimulation above control

MAPK-P/Total MAPK ratio

60 kDa
Figure 4

| Table 2 |
|-----------------|-----------------|-----------------|-----------------|
|               | Control | IGF-I 100 nM | IGF-I 100 nM + Wortmannin (1µM) | IGF-I 100 nM + PD-98059 (50 µM) |
| MAPK-P         | 100 %\(^{a}\) | 171 ± 29 %\(^{b}\) | 118 ± 9 %\(^{a}\) | 51 ± 13%\(^{c}\) |
| Akt-P          | 100 %\(^{a}\) | 196 ± 5 %\(^{b}\) | 89 ± 8 %\(^{a}\) | 191 ± 36 %\(^{b}\) |