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PAPER DE LA INSULINA I FACTORS DE CREIXEMENT TIPUS INSULINA EN LA REGULACIÓ DEL CREIXEMENT I METABOLISME EN TRUITA I ORADA

Tesi Doctoral

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L'estimulació dels IGFs en cèl·lules musculars d'orada (*Sparus aurata*) en cultiu té lloc degut a l' acció específica del receptor d' IGF-I i de les vies MAPK i PI3K.

IGFs stimulate gilthead sea bream (*Sparus aurata*) cultured muscle cells through its specific type I receptor by MAPK and PI3K-kinase-signaling pathways

L'estimulació dels IGFs en cèl·lules musculars d'orada (Sparus aurata) en cultiu té lloc degut a l' acció específica del receptor d'IGF-I i de les vies MAPK i PI3K/Akt.

Resum

En el present treball s'han analitzat les variacions en el binding d'IGF-I al llarg del desenvolupament *in vitro* de cèl·lules musculars d'orada en cultiu.

La viabilitat ha estat determinada mitjançant la tècnica del FACS (fluorescent activated cell sorting) i l'assaig del MTT. L'anàlisi per FACS ha mostrat que el percentatge d'apoptosis disminueix en funció de la diferenciació cel·lular, essent menys del 5 % a partir del dia 4 en el cultiu. Per una altra banda, s'han observat increments en la capacitat de les cèl·lules en reduir el compost MTT a formazan a partir del moment en que les cèl·lules començaven a diferenciar-se (dia 4).

Una vegada es va confirmar el fenotip de les cèl·lules musculars d'orada, es van realitzar estudis de binding d'IGF-I en dos estadis del desenvolupament cel·lular: miòcits (dia 5) i llargs miotubs (dia 12). Els experiments de binding es van dur a terme incubant les cèl·lules en presència d' IGF-I durant 16 hores a 4º C. El número de receptors (R_0) va incrementar de 190 ± 0, 09 (fmol/mg proteïna) en el dia 5 de cultiu a 360± 0, 09 (fmol/mg proteïna) en el dia 12. L'afinitat (Kd) dels receptors d'IGF-I no va patir canvis significatius al llarg del desenvolupament (de 0, 89± 0, 09 en el dia 5 a 0.98± 0, 09 nM en el dia 12).

Es van estudiar els efectes dels IGFs i de la insulina en l'activació de les vies de senyalització ERK 1/2 MAPK i Akt/PKB, mitjançant la tècnica del western blot. La fosforil·lació de la MAPK ha incrementat degut als efectes dels IGFs durant les primers fases del cultiu, però es va observar una resposta molt baixa en el dia 15. Contràriament, els IGFs han estimulat la fosforil·lació de l'Akt al llarg de tot el cultiu.

En conclusió, aquest estudi mostra que les cèl·lules musculars d'orada en cultiu, expressen un receptor d'IGF-I funcional que incrementa a mesura que es diferencien les cèl·lules. Igualment, s'ha demostrat que els IGFs a través de la unió al receptor d'IGF-I, activen la via de la MAPK i de l'Akt, en funció de l'estat de desenvolupament cel·lular en el cultiu.

IGFs stimulate gilthead sea bream (*Sparus aurata*) cultured muscle cells through its specific type I receptor by MAPK and PI3-kinase-signaling pathways

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Abstract

The present study examined the variations of IGF-I binding during the *in vitro* development of gilthead sea bream muscle cells. The viability of the cell culture was determined by fluorescent activated cell sorting (FACS) analysis and MTT assay. FACS analysis showed that the percentage of death cells decreased with the cell differentiation, being less than 5 % of mortality since day 4 to the end of the cell culture. On the other hand, measured by optical density (OD), intracellular reduction of MTT into formazan pigment formation, was preferently carried out as cells differentiated (from day 4). Having successfully confirmed the phenotype of gilthead sea bream muscle cells, IGF-I binding assays were performed in two stages of cell development: myocytes (day 5), and large myotubes (day 12). Binding experiments were done by incubating cells with IGF-I for 16 h at 4°C. The number of receptor (R_0) increased from 190± 0, 09 fmol/mg protein at days 5 to 360± 0, 09 fmol/mg protein on day 12. The affinity (Kd) of IGF-I receptors did not change significantly during the cell culture development (from 0, 89± 0, 09 to 0.98± 0, 09 nM). By means of western blot analysis the activation of ERK 1/2 MAPK and Akt/PKB proteins by IGFs was studied. MAPK-P was increased by IGFs in the first stages of the cell culture, but low response was observed at day 15. Contrary, IGFs displayed a stimulatory effect on Akt-P throughout all the cell culture, even on day 15.

In conclusion, this study shows that primary culture of gilthead sea bream myocytes express functional IGF-I receptor which increased in number as skeletal muscle cells differentiated in culture. It is also demonstrated that IGFs signaling transduction through IGF-I receptor stimulates MAPK and Akt pathways depending on the development stage of the muscle cell culture.

1. Introduction

Among vertebrates, the insulin-like growth factors (IGFs) are growth promoting polypeptides which play an essential role in growth and development (Yakar et al., 2005). Both IGF-I and IGF-II are the only known growth factors which stimulate proliferation and differentiation processes in muscle cells (Reviewed by Oksbjerb et al., 2004) and are critically involved in skeletal muscle development (Florini et al., 1991). During the activation of satellite cells, also termed myoblast (Ishido et al., 2003), all these factors directly regulate most of the biological actions through the IGF-I receptor (IGF-IR) contributing to the muscle growth and development (Napier et al., 1999). Few studies using cultured fish cells showed that the activation of the IGF-IR is linked to two major intracellular signaling pathways, the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3 kinase)/protein kinase B (PKB also called Akt) (Pozios et al., 2001; Castillo et al., 2006), like reported in mammals by Valentinis et al. (2001).

The binding of IGF-I to its receptor elicits different biological responses, including cell differentiation, proliferation and survival mechanisms (Wu et al., 2004). Our group was the first in characterize the binding for IGF-I in primary cell culture of trout myocytes (Castillo et al., 2002), as well as the mitogenic and anabolic effects of insulin and IGF-I throughout the cell culture (Castillo et al., 2004). These studies reveal that the PI3-kinase pathway plays a significant role in mediating IGFs action on 'metabolic' responses, whereas the MAP kinase pathway is also thought to mediate the 'mitogenic' effects of IGFs (Castillo et al., 2006).

The critical role of IGF system in somatic growth has been established in mice models where the IGFs and their receptors have been ablated. In this way, IGF-I receptor null-mice displayed extremely retarded growth and died at birth, due to the poor organ development (Liu et al., 1993). Furthermore, Cheng et al. (2000) found out that functional inactivation of endogenous IGF-I receptors in C2C12 cells skeletal myoblasts, abolished IGF-I-induced proliferation and resulted in a delay in the differentiation process. The behaviour of the IGF-I receptor during differentiation depends on the cell source (Reviewed by Oksbjerg et al., 2004). We have previously reported an up-regulation for the IGF-I receptors from proliferation to differentiation stages in rainbow trout satellite cells (Castillo et al., 2002). This effect has been also reported in C2C12 mouse satellite cell line derived from adult mouse (Tollefsen et al., 1989) and in human muscle cells (Crown et al., 2000). In contrast, studies on L6 (cell

line isolated from the thigh muscle of prenatal rats), BC3H-1 (line from mouse) and on turkey satellite cells, indicated that the number of IGF-I receptor is down-regulated in differentiation (Benguinot et al., 1985; Rosenthal et al., 1991; Minshall et al., 1990).

The culture of trout myocytes described by Rescan et al. (1995) and lately optimized by Faconneau and Paboeuf (1998), has been used as an useful tool in order to analyze the regulation of IGFs and its receptors in trout muscle cultured cells (Castillo et al., 2002). Fish models can contribute much more to our knowledge about the role of the IGF system in muscle growth and development, which is important considering the fact that fish have continuous growth (Reviewed in Mommsen, 2001), and therefore IGF-I could act as a key regulatory factor of this growth through the fish life cycle. On the other hand, studies undertaken by Perrot et al. (1999, 2000) showed that IGF-IR and their ligands (IGF-I and IGF-II) were expressed during early development stages and gonad development in the gilthead sea bream.

The gilthead sea bream is a widely fish cultured in Southern Mediterranean area, but basic research is still needed in order to understand the mechanisms involved in the process of somatic growth. In the present study we aimed to develop and perform a primary cell culture of muscle gilthead sea bream cells in order to characterize IGF-I binding and the evolution of IGF-I receptors during myocyte differentiation. Our data showed that the PI3K/Akt and MAPK pathways lead the biological responses of the binding of IGF-I peptide to its receptor. This study represents the first step to understand the role of these receptors in the gilthead sea bream physiology.

2. Materials and Methods

2.1. Animals and experimental conditions

We used gilthead sea bream (*Sparus aurata*) with weights ranging from 2.8 to 8.5 g. These fish were maintained in Barcelona facilities in closed-circuit flow systems at 19°C, fed *ad libitum* with a commercial diet, and fasted for 24 h before the experiments. The fish (70 to 80 for each culture) were killed by a sharp blow to the head, weighted and immersed in 70% ethanol for 30 s to sterilize external surfaces. For binding analysis in white skeletal muscle, 15 animals were killed by a blow to the head and white skeletal muscle was excised and frozen at – 80 ºC until further analysis.

2.2. Isolation of myosatellite cells

The protocol described by Fauconneau and Paboeuf was subjected to some modifications as follows. White myotomal muscle was excised under sterile conditions and collected in cold (4°C) DMEM, 9 mM NaHCO3, 20 mM HEPES (pH 7.4, 360 mosmol/kgH2O), containing 15% horse serum and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml, fungizone 0.25 µg/ml, gentamycin 75 µg/ml) at a concentration of 5 g/ml. The tissue was minced using sterile blades and fragments were centrifuged (300 g, 5 min) and washed twice in DMEM without horse serum to eliminate the serum components. Enzymatic digestion was performed with collagenase (Type Ia Sigma) in DMEM (final concentration 0.2%) for 80 minuts at 21°C with gentle agitation. The suspension was centrifuged (300 g, 5 min), and the pellet was washed with DMEM, resuspended in DMEM (5 ml/g of muscle), triturated through a pipette five times, and centrifuged again (300 g, 5 min). Fragments were resuspended in a trypsin solution 1:250 (Sigma) (0.1% final concentration in DMEM) for 20 min at 21°C with gentle agitation and centrifuged for 1 min at 300 g. The supernatant was diluted in 2 vol of cold complete medium to block trypsin activity. The tissue fragments (pellet) were given a second similar trypsin digestion, and the suspension was then diluted. The two supernatants of digestion were pooled, diluted (1:1 vol/vol) in cold DMEM supplemented with 15% horse serum, and centrifuged (300 g, 20 min, 10°C). The resulting pellets were resuspended in 30 ml of basal medium and submitted to mechanical dissociation by trituration five times with a 10-ml pipette and five times using a 5-ml pipette. The suspension was then filtered successively on a 100-µm and 40-µm nylon cell strainer and centrifuged (300 g, 20 min, 4°C). The cells were resuspended in basal medium supplemented with 10% fetal calf serum (FCS) and diluted to reach a final concentration of 1,5 x 10^6 cells/ml of normal medium.

2.3. Cell culture

Culture plates were pre-treated with a solution of 100 µg/ml of poly-L-lysine (Sigma MW300000) at a concentration of 16 µg/ cm2 for 2 h 30 min at 21°C. After this precoating, the poly-L-lysine solution was aspirated, substratum was washed twice with sterile miliQ® water, air dried and closed until the plating of the cell suspension, which contained a crude extract of muscle cells. Culture was performed with this crude extract, without selection of satellite cells. Cells were cultured in complete medium at 18°C in air, in 6 well plastic plates (9.6 cm2/well, NUNC), and medium was changed every

days. For the MTT and flow citometry assays 0, 5 x 10^6 cells/ ml of cells were cultured in 24 well plastic plates. Observations of morphology were regularly made to control the state of the cells.

2.4. MTT assay

MTT reductase assay was used as a measure of functional mitochondrial dehydrogenases on isolated gilthead sea bream myocytes. The formation of a blue formazan product, due to reduction of the yellow MTT tetrazolium in salt by mithocondrial reductases, was quantified in isolated gilthead sea bream myocytes by spectrophotometry. Under sterile conditions, 100µl of MTT (5mg/ml) (Sigma Aldrich, Spain) was added to each well containing basal medium supplemented with 10% fetal calf serum (FCS) DMEM. Plates were recovered with aluminium-foil, and incubated at 21ºC for 16 hours. Untransformed MTT that remained in the solution bathing myocytes was removed and wells were washed twice with sterile PBS. The formazan product was dissolved in DMSO and cells were removed scrapping gently. Cells without MTT incubated with DMSO were used as negative control. After the incubation period, assays were measured at 540 nm wavelength using Beckman DU64 spectrophotometer.

2.5. Flow citometry

Cells (0,5x 10^6) were washed twice with DMEM medium and placed in a tube containing 40µg of propidium iodide for few minutes in the dark. Staining for DNA content was measured by flow citometry. Samples were analyzed on FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), counting 10^4 live cells and using CellQuest software (Becton Dickinson).

2.6. IGF-I binding assays in cells

Binding studies were conducted with cells seeded at a density of 1.5 to 2 x10^6 per well. In each well, monolayers were washed three times with 2.5 ml of cold PBS and incubated for 16 h at 4°C in 1 ml DMEM containing 0.5% BSA (free of insulin, A-7888 Sigma) with ^{125}I-labeled rhIGF-I (Amersham Pharmacia Biotech Europe GmbH, Barcelona, Spain) in the presence or absence of a range of concentrations of cold peptide (from 1 to 1000 ng/ml of rhIGF-I). Non-specific binding (NSB) was obtained with a concentration of 400 ng/ml of cold rhIGF-I.

Incubation was stopped by aspiration of medium. Next the monolayer was washed twice with 2.5 ml of cold PBS, and cells were triturated by incubation with 1 ml 0,1 N NaOH for 30 min at RT. Radioactive solution was counted using a gamma

counter (Packard Bioscience, Meriden, CT). Each binding experiment was performed in duplicate at least three times for each developmental stage. The protein content of each well was determined following the method described by Bradford (1976). Specific binding (SB) is calculated as follows: SB= TB – NSB, where the (NSB) is the non specific binding and (TB) the total binding.

2.7. IGF-I binding assay in white skeletal muscle

Partial purification of solubilized IGF-I receptors from white muscle was performed at 4 °C, as described by Párrizas et al. (1995) by affinity chromatography on wheat-germ agglutinin (WGA) bound to agarose (Vector Laboratories, Burlingame, USA). The glycoproteins obtained were measured following the method described by Bradford (1976). Binding assays were performed as in Párrizas et al. (1994). A volume of 30–40 µl of the WGA eluate (approximately 30 µg of glycoproteins) was incubated for 14–16 h at 4 °C with increasing concentrations of cold hormone (from 0 to 100 nM, final dilution) and the radiolabelled ligand as tracer (25 pM). Semi-purified receptors were precipitated by addition of 0.08% bovine γ-globulin and 10.4% polyethylene glycol (final concentrations), followed by centrifugation at 14,000×g for 7 min at 4 °C. Binding data were analysed in Scatchard plots and only the high-affinity, low-capacity binding sites were considered in the analysis. Recombinant human IGF-I was purchased from Peninsula Laboratories, Europe (Merseyside, UK). Human recombinant 3-^{125}I-IGF-I, both with 2000 Ci/mmol specific activity, were purchased from Amersham Life Sci. (Amersham Pharmacia Biotech Europe GmbH, Barcelona, Spain). All other chemicals used were purchased from Sigma Aldrich Química (Alcobendas, Madrid)

2.8. Western blot analysis

In order to determine the intracellular signal transduction pathways activated by insulin and IGF-I and IGF-II during in vitro development of gilthead sea bream skeletal muscle cells in culture, Western blots against several proteins involved in the PI3K-Akt and MAPK pathways were performed. Following 5, 8 or 13 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 and IGF-II or 1µM 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 loaded with 30 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 for 1 hour at RT 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). 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).

2.9 Statistical analysis

The treatment was performed in duplicate in each experiment. All data are presented as means ± SE of at least three experiments (Scatchard assays). Statistical differences between conditions were tested by ANOVA. Differences were considered statistically significant at $P < 0.05$.

RESULTS

3.1 Cell culture progression and viability

Cell cultures were visualized by phase contrast light microscopy (X 40 magnification). The phenotype of cells in different days of culture is shown in Fig. 1: in day 1, cells are mononucleated (Fig.1A), and throughout their development they fuse to form small myotubes (day 5; Fig. 1B) and large myotubes later on (day 15; Fig. 1C).

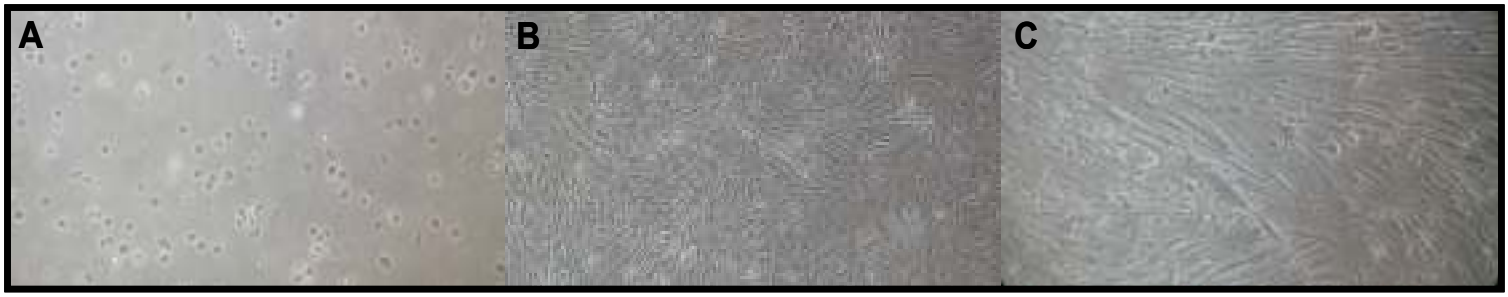

Fig. 1. Differentiation of satellite cells isolated from skeletal muscle of juvenile gilthead sea bream and cultured on a laminin substrate with DMEM 90% / fetal calf serum 10% medium at 21°C for 1 day (*A*), 5 days (*B*), and 13 days (*C*).

By means of MTT reduction assay it is found that morphological differentiation into myotubes was accompanied by a three-fold increase in the reduction of MTT to formazan from day 1 to day 15 in the cell culture (Fig. 2).

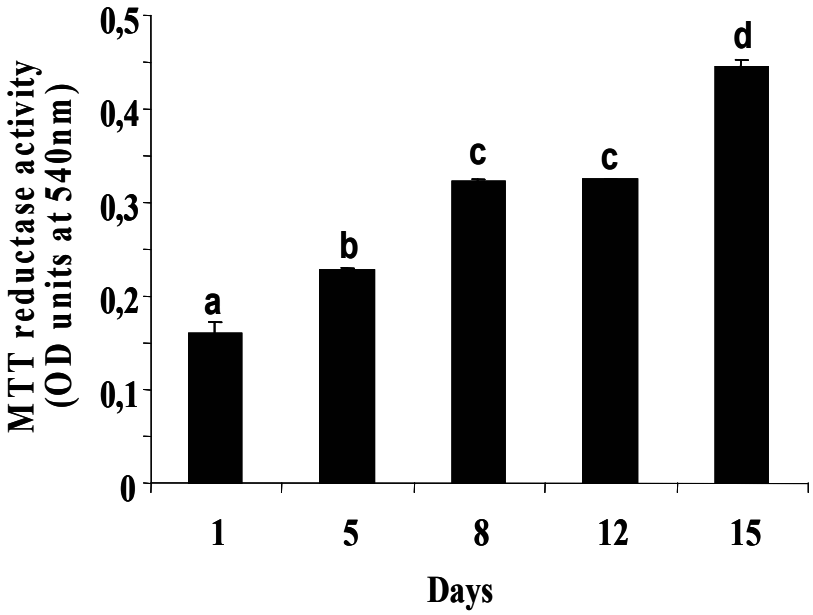

Fig. 2. Cell mithocondrial viability was determined using the MTT conversion assay.. Data are means ±S.E.M of 3 separate experiments, each performed in duplicate. Different letters indicicate significantly different values at $P < 0.05$.

Cell viability was measured by fluorescent activated cell sorting (FACS) analysis. As shown in Fig. 3A, the percentage of death cells decreased as the cells differentiate and fuse in large myotubes, being less than 5 % of mortality from day 4 to the end of the cell culture. The distribution in the cell population by the morphological features are represented in Fig. 3B by forward light scatter (FSC-H), accounting for cell dimension, and side light scatter (SSC-H), accounting for cell condensation and granularity at 5, 8, 12 and 15 days gilthead sea bream muscle cells. As indicated in the plots the gilthead muscle sea bream population is well distinguished throughout the cell

culture and progresses in a time dependent manner. From day 5 in the cell culture the population begins to differentiate (cells marked by a triangle). As long as differentiation takes place, gains of cell dimension (FSC-H), condensation and granularity (SSC-H) are observed (Fig. 3B).

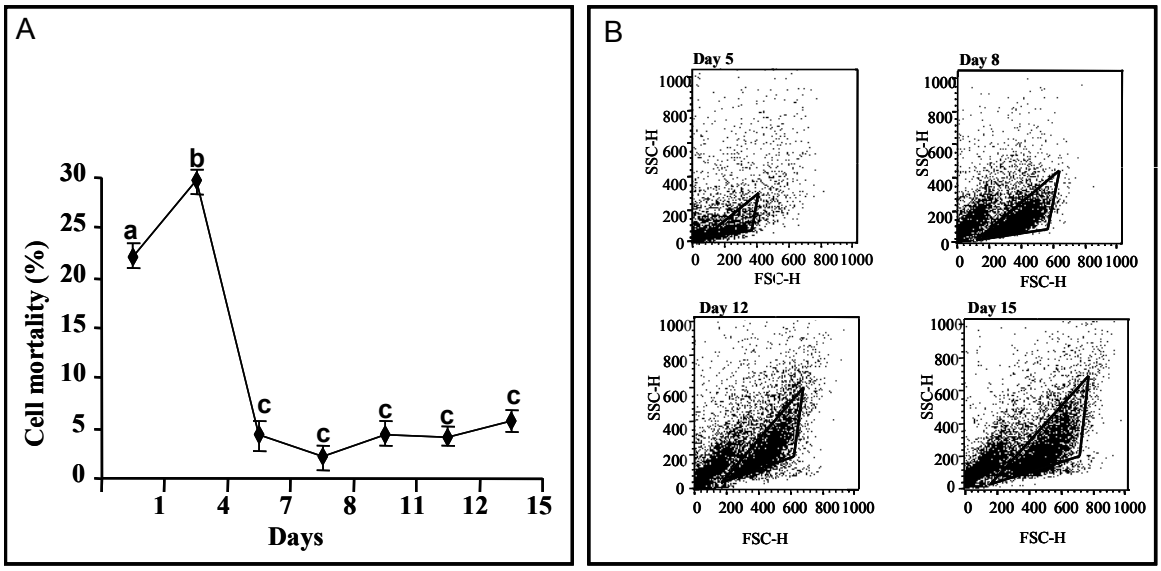

Fig. 3. Apoptosis was analyzed by fluorescence-activated cell sorting throughout the cell culture.
(A) Percentage of specific apoptosis was determined after incubating cells with propidium iodide for few minutes and subsequent analysis on 10^4 cells of the surrounded area at each day indicated of the cell culture development. Data are means ±S.E.M of 3 separate experiments, each performed in duplicate. Different letters indicicate significantly different values at $P < 0.05$
(*B*) Distribution in the cell population of the morphological features represented by forward light scatter (FSC-H), accounting for cell dimension, and side light scatter (SSC-H), accounting for cell condensation and granularity at 5, 8, 12 and 15 days gilthead sea bream muscle cells.

3.2. IGF-I binding

Figure 4 shows a plot of the IGF-I binding to muscle cells at day 12 of the cell culture. We observed that using non-labelled human IGF-I with increasing concentrations (ranging from 1 to 1000 ng/ml), displacement of ^{125}I - rhIGF-I was assessed.

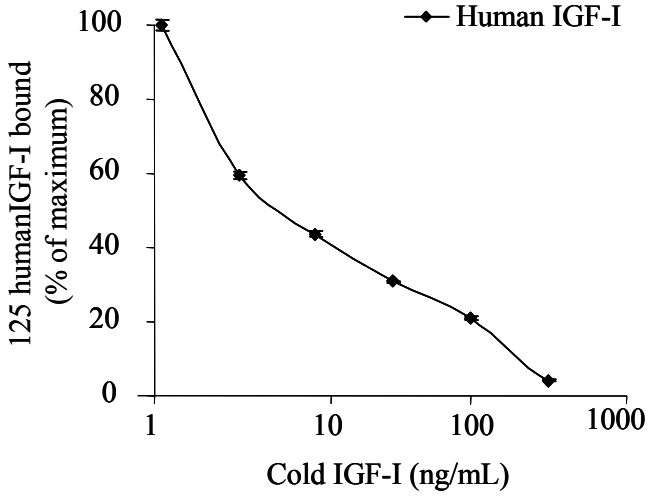

Fig. 4. Human IGF-I binding to 12- days muscle cells. Cells were incubated for 16 h at 4°C with a fixed amount of labeled trout IGF-I and increasing concentrations of cold human IGF-I. Data are means ±S.E.M of 3 separate experiments, each performed in duplicate. Different letters indicicate significantly different values at P <0.05.

The Scatchard transformation of the specific binding curve provided, together with the specific binding, the number of receptors (Ro) and their affinity (K_d) (Table 1) at the two different cell stages analyzed: day 5 (small myotubes) and day 12 (large myotubes). In the table are shown results of the same parameters obtained from muscle glycoproteins homogenates of these animals. Receptor affinity did not show significant changes at the different stages of the cell culture analyzed (K_d from 0, 89 to 0, 98). The number of IGF-I receptors indicated an increase from day 5 to day 12 in the cell culture (from 0, 19 to 0, 36 pmol/mg protein). When glycoprotein homogenates from the muscle of these animals were analyzed, the number of receptors and their affinity were in the same range as those obtained from the preparations of the isolated cells.

Table 1. Binding characteristics of IGF-I receptors in gilthead sea bream muscle cells and muscle glycoprotein homogenates.

	Kd (nM)	**Ro(pm/mg)**
Day 5	**0,89±0.38**	**0,19±0.09^{a}**
Day 12	**0,98±0.3**	**0,36±0.09^{b}**
Skeletal muscle	**0,81±0,8**	**0,18±0,03^{a}**

Data are means ±S.E.M of 3 separate experiments, each performed in duplicate. Different letters indicicate significantly different values at P <0.05.

Specific binding in culture myocytes ranged from 14, 82 ± 1, 9 % to 24, 85 ± 1, 2 % increasing throughout the cell culture (Figure 5). Binding in glycoprotein homogenates from the muscle of these animals was 16 ± 3, 9 %.

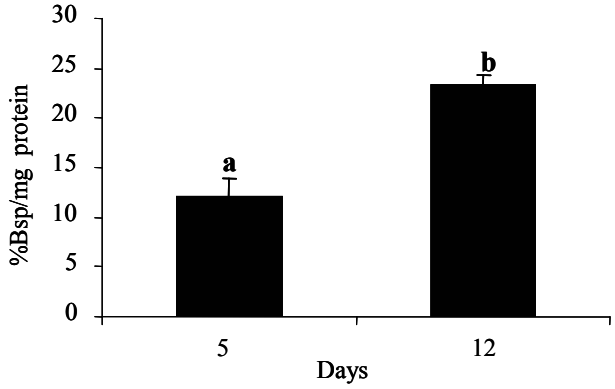

Fig. 5. Specific binding values of IGF-I purified receptor preparations of gilthead sea muscle cells at day 5 and 12 in the cell culture. Data are means ±S.E.M of 3 separate experiments, each performed in duplicate. Different letters indicicate significantly different values at P <0.05.

3.3. Insulin and IGF-I signaling pathways in skeletal muscle

The activation of different IGF-I, IGF-II and insulin receptor signal transduction pathways was determined by Western blot. We analyzed the presence of both total and phosphorylated MAPK and Akt proteins throughout the different developmental stages of the gilthead sea bream skeletal muscle cells in a primary culture. These Western blots were performed from lysates of gilthead sea bream skeletal muscle cells on day 5 and day 8 myoblasts (small or bigger myotubes formed by fusion of few cells) and large myotubes on day 15.

MAPK pathway.

Figure 6 A shows the effects of peptides at each stage of development, normalized with the total MAPK content (MAPK-P/ MAPK). In myoblasts on day 5, all the analyzed peptides (insulin, IGF-I and IGF-II) caused an almost 4, 5-fold increase in MAPK-P levels comparing with MAPK-P basal levels. At day 8 MAPK-P levels were slightly lower stimulated in the presence of the peptides; the stimulation due to the effect of IGFs was higher than with insulin (380 % for insulin, 420 % for IGF-I and 510 % for IGF-II). In differentiated myotubes (day 15) the response to stimulation was lower than at 8 and 5 days.

PI3K-Akt pathway

Figure 6 B reveals that on day 5, insulin and IGFs caused from 2 to 3-fold increase in Akt-P levels (normalized with total Akt). At day 8 in the cell culture the increase for Akt-P levels was higher for IGFs compared to insulin (~300% vs. 450%, respectively). On day 15 both peptides still caused a significant increase in Akt-P levels, clearly higher for IGF-II than insulin or IGF-I (550%, vs~400% respectively).

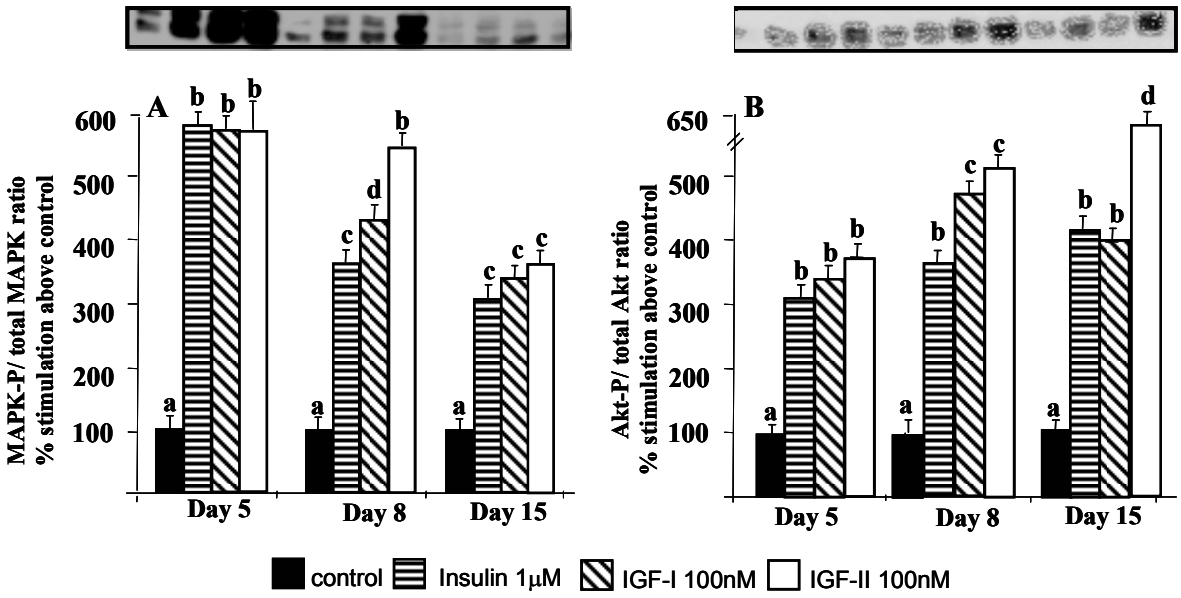

Fig. 6. Stimulation of MAPK (A) and Akt phosphorylation (Akt-P) (B) by insulin (1μM) and IGFs (100 nM) in gilthead sea bream muscle cells at different stages of development. Cells were incubated with peptides for 30 minutes, lysed, 30 μg of protein were loaded in each lane and subjected to a 10% SDS-PAGE under reducing conditions. Densitometry analysis of three independent experiments, normalized for the content of total MAPK or Akt, respectively is shown. Different letters indicate significant differences ($P<0.05$).

4. Discussion

Culture of muscle satellite cells have been developed for different fish species (Powell et al., 1989; Sepich et al., 1994; Matschak and Stickland, 1995; Cook et al., 1995), but only in few studies the satellite cells undergo into very large myotubes as a model of primary cell culture (Rescan et al., 1994; Fauconneau and Paboeuf, 2000; Castillo et al., 2002). The lack of these studies in marine fish species led us to develop and establish a primary cell culture of gilthead sea bream myocytes. In the current study we report the presence of specific IGF-I receptors throughout the cell culture as well as the main signaling pathways involved in IGF-I receptor signaling transduction. The

stimulatory effects of insulin and both IGF-I and IGF-II on these signaling pathways have been assessed.

In this *in vitro* model, it was found that large myotubes appeared at day 8 in the cell culture, and that very large myotubes were present up to day 15. There is a certain delay in comparison with rainbow trout cultured muscle cells, where, Castillo et al (2002), showed that at 4 day myocytes developed to large multinucleated myotubes at day 11 in the cell culture. The morphological differentiation of gilthead sea bream myocytes is well correlated with that found by Al-Khalili et al. (2004), where human cultured myocytes undergo to large myotubes at day 14 in the cell culture.

Cell viability and proliferation are important parameters to take into account when assessing primary cell cultures (Greenle et al., 1995; Fauconneau and Paboeuf, 2000; Pozios et al., 2000; Castillo et al., 2004). The present study showed two non-radioactive methods for measuring cell viability in sea bream muscle cells: MTT assay and fluorescent activated cell sorting (FACS) analysis.

The MTT reductase assay measures formazan product formed by functional mitochondrial dehydrogenases on isolated gilthead sea bream myocytes. In the current study, the increases in MTT reductase activity throughout the cell culture evidenced that the development of the cell culture promoted cell growth under the present conditions.

Cellular adhesion has been correlated in keeping high MTT reductase activity in viable cells with active mithocondrial function (Klein et al., 1981; Sladowski et al., 1993). In this context, we suggest that the fusion of myocytes to large myotubes permits the assessment of cell viability, which increased as well as cell go on differentiation and fusion. On the other hand, cell number and viability has been studied by the incorporation of propidium iodur into nuclei of sea bream muscle cells at different days on the cell culture. By FACS analysis, death cells can be easily distinguished throughout the cell culture and it has been showed that cell death clearly decreases and is maintained lower than 5 % until day 15 in the cell culture. In the same way, the presented data coincides with other studies (Sladowski et al., 1993), where cell viability increases as long as the cell culture developed.

As a first time in this species we detected the presence of specific IGF-I receptors throughout the cell culture. Furthermore, the specific binding (SB) was in a range from ~10 to 20 % throughout the cell culture, indicating that SB increased with cell differentiation. Recently, Castillo et al. (2002) showed a SB ~3% in rainbow trout myosatellite cells. The higher SB values reported in sea bream myocytes could be

related with the elevated plasmatic values for IGF-I in this species comparing with those reported in fresh water species (Pérez-Sánchez et al., 1994, ; Mingarro et al., 2002; Gómez-Requeni et al., 2004, 2005). On the other hand, higher SB values could be related to the reported high amounts of IGF-I receptors in muscle preparations of marine fish species when comparing with fresh water species (Reviewed in Navarro et al., 1999). In the current study, when SB is analyzed in muscle preparations of gilthead sea bream, we detected that the percentage of hormone binding to its receptor is in the same range as that found in *in vitro* cultured cells and also in previous studies undertaken in white skeletal muscle of different fish species (Párrizas et al., 1995; Baños et al., 1998), confirming that this *in vitro* system offers a suitable model for the study of the IGF-I receptor binding properties. In the sea bream cell culture we did not find changes in the affinity (Kd) for the receptor throughout development from proliferation to differentiation. These results confirm data described by other authors using different muscle cell culture models (Benguinot et al., 1985; Tollefsen et al., 1989; Minshall et al., 1990; Rosenthal et al., 1991; Ernst et al., 1999; Crown et al., 2000; Castillo et al., 2002). Thus, the data reported in the present study suggest that increases in IGF-I receptor number and SB might be related with the differentiation of the primary gilthead sea bream myocytes. In fact, functional inactivation of the IGF-I receptor delays differentiation of C2C12 muscle cells (Cheng et al., 2003), and its overexpression accelerates myogenic differentiation (Quinn et al., 1994; Quinn and Haugh 1996).

It has been described that IGFs stimulate muscle cell proliferation and differentiation in culture through interaction with the IGF-I receptor (Ewton et al. 1987; Duclos et al., 1991; Galvin et al., 2003). IGFs binding to IGF-I receptor stimulates tyrosine kinase activity and activation of the mitogen-activated protein (MAP) kinase signaling pathway during proliferation (Coolican et al., 1997) and differentiation (Wu et al. 2000), in addition to activation of the phosphoinositide 3'-kinase (PI3K)/Akt pathway during differentiation (Coolican et al., 1997). In the present study the phosphorylation of MAPK in the presence of insulin, but especially of IGFs, was highly increased in small myotubes (day 5), being less marked at (day 8) and week at day 15 in the cell culture. In mammals IGF-I stimulated proliferation of L6A1 myoblasts activating the MAPK pathway (Coolican et al., 1997). Insulin has been described to act in the same pathway in human muscle cultured cells (Al-Khalili et al., 2004). In fish, studies in embryonic zebrafish ZF-4 cell line described the effects of IGF-I and IGF-II in the activation of the MAPK pathway during proliferation and early differentiation

(Pozios et al., 2001). More recently, Castillo et al. (2006) reported the higher response of MAPK-P in early stages of development of rainbow trout muscle cultured cells incubated with insulin or IGF-I, pointing out the role of this pathway in myocyte proliferation.

On the other hand, Akt phosphorylation (Akt-P) increased in the presence of insulin, but especially IGFs, in both myotubes (day 8) and large myotubes (day 15), being less marked at day 5 (myocytes). Our data are well correlated with that found in mammalian studies on muscle cell cultures, where the active Akt-P was increased in presence of insulin or IGF-I in differentiated myotubes or large myoblasts (Coolican et al., 1997; Al-Khalili et al., 2004). In fish, Pozios et al. (2001) reported increases in Akt-P in the presence of IGF-I and IGF-II in ZF-4 cell line. In the same way, Castillo et al. (2006) described similar results when rainbow trout muscle cells were incubated with insulin and IGF-I. It has been widely demonstrated that cell growth and survival in response to insulin and IGF-I is critically mediated by Akt (Stitt et al., 2004). The current study clearly showed that insulin, but especially IGFs increase Akt-P during differentiation. Increases in Akt-P in the presence of insulin and IGFs coincide with cell mortality decrease since day 5 in the cell culture. Thus, like described recently by Wu et al. (2004) in PC-12 cells, it seems that the PI3-kinase/AKT pathway appears to be correlated in the inhibition of apoptosis by IGFs action in gilthead sea bream muscle cells (Wu et al., 2004).

In conclusion, this study firstly characterizes the development from mononucleated myoblasts to polinucleated gilthead sea bream myotubes. Secondly, we set out by fluorescent activated cell sorting (FACS) analysis and MTT assays, that cell viability is accompanied by increases in the specific binding and number of IGF-I receptor. In the other hand the PI3-kinase/AKT pathway, is clearly activated in medium and final stages of the cell culture, suggesting an essential role of this pathway in the differentiation and survival stages, whereas the MAPK pathways seems to be implicated on myocyte proliferation. The results obtained in the present work encourage us to further investigate the different role of insulin and IGFs on gilthead sea bream muscle cells metabolism and growth.

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