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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

Núria Montserrat Pulido

Efectes metabòlics de la insulina i dels IGFs en cèl·lules musculars de l'orada (*Sparus aurata*) en cultiu.

Metabolic effects of insulin and igfs on Gilthead Sea Bream (*Sparus aurata*) muscle cultured cells.

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Resum

S'ha analitzat la funció metabòlica dels IGFs de manera comparativa amb la de la insulina en cèl·lules musculars d'orada al llarg del desenvolupament del cultiu.

S'han estudiat els efectes *in vitro* de la insulina i dels IGFs sobre la captació de 2-Deoxy-Glucosa (2-DG) i de L-alanina en miòcits (dia 4) i petits miotubs (dia 9). La captació de 2-DG incrementa en presència de la insulina, però en major grau, dels IGFs. L'estimulació de la captació de 2-DG és depenent del temps d'incubació i de l'estat de diferenciació de les cèl·lules. Per contra, la captació de L-alanina va mostrar un patró de desposta invers, essent major quan les cèl·lules musculars estan poc diferenciades (dia 4).

La incubació de les cèl·lules amb PD-98059 o wortmanina, però especialment de citocalasina B, va provocar una disminució en la captació de 2-DG en miòcits (cèl·lules a dia 5), suggerint que el transport de glucosa és degut a l'acció de transportadors de difussió facilitada. Els extractes proteics de cèl·lules preincubades amb wortmanina i PD-98059, i posteriorment amb els pèptids, van ser analitzats per Western blot. S'ha detectat que l'activació de les vies de transducció de la MAPK i de l' Akt estan implicades en els efectes metabòlics dels IGFs. De la mateixa manera, s'ha detectat que la insulina i els IGFs estimulen la síntesis proteïca del transportador GLUT4 durant el desenvolupament del cultiu.

El present treball mostra que els IGFs estimulen la captació de 2-DG i de L-alanina en major grau que la insulina. De la mateixa manera, el grau d'estimulació dels pèptids varia en funció de l'estat de diferenciació de les cèl·lules. Els efectes metabòlics dels IGFs es donen a través de l'activació de les vies de transducció de la MAPK i de l'Akt. A més, els canvis en la captació de glucosa poden ser explicats per l'acció dels transportador GLUT4, que és estimulat en presència dels pèptids al llarg del cultiu. Aquests resultats indiquen el paper dels IGFs en el metabolisme de les cèl·lules musculars d'orada.

Metabolic effects of insulin and IGFs on Gilthead Sea Bream (*Sparus aurata*) muscle cultured cells

Montserrat N., Codina M., Navarro M.I. and Gutiérrez J.

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, E-08028 Barcelona, Spain.

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Corresponding Author: Dr Joaquim Gutiérrez Fruitós

Corresponding Author's Institution: Faculty of Biology, University of Barcelona

Abstract

The relative metabolic function of insulin compared with IGF-I and IGF-II on gilthead sea bream muscle cells, has been investigated at different stages in the cell culture. In these cells the *in vitro* effects of insulin and IGFs on 2-deoxyglucose (2-DG) and L-alanine uptake was carried out in myocytes (day 4) and little myotubes (day 9). 2-DG-uptake in gilthead sea bream muscle cells was increased in the presence of insulin and IGFs in a time dependent manner and as well as the cell culture progressed and differentiated. On the contrary, L-alanine uptake showed an inverse pattern, being higher in little myocytes than in large myotubes. When incubating the cells with PD-98059, wortmannin and especially cytochalasin B, a reduction in 2-DG uptake was observed, suggesting that glucose transport takes place through specific facilitative transporters. By means of Western blot on lysates from cells preincubated with the specific inhibitors and subsequently stimulated with insulin and IGFs, we detected that MAPK and Akt transduction pathways are implicated in the metabolic function of the peptides. In the same way, we detected that GLUT4 protein synthesis is stimulated in the presence of insulin and IGFs in gilthead sea bream muscle cells in a different manner throughout the cell culture.

In conclusion, this study show that IGFs are more effective than insulin in stimulating glucose and alanine uptake in gilthead sea bream myosatellite cells and that the degree of stimulation changes when cells differentiate to myotubes. The metabolic actions of the IGFs activate the MAPK and Akt transduction pathways. Moreover, changes in glucose uptake can be explained by the GLUT4 transporter action, which is stimulated in presence of the studied peptides throughout the cell culture. These results indicate the important role of IGF-I and IGF-II on the muscle metabolism of the gilthead sea bream

1. Introduction

The farming of gilthead sea bream, *Sparus aurata*, is one of the most important aquaculture industries in the Mediterranean area (Mingarro et al., 2002). In the last decades the regulation of growth is one of the goals of aquaculture production (Pedroso et al., 2005). Considering that muscle in fish can account for about 60% of the weight of an adult individual (Millward et al., 1989), and that muscle growth will determine the final size of the animal, the adoption of practices for manipulation of fish growth is conditioned to the understanding of muscle growth regulating processes (Cyrino and Mulvaney, 1999).

Post-larval growth in fish results from both an increase in diameter of already existing fibers (hypertrophy) and the recruitment of new fibers (hyperplasia) (Rowerlson and Vegetti, 2001). The hyperplasic phenomenon continues indeterminantly beyond puberty (Weatherley et al., 1987; Mommsen and Moon, 2001) and results of the recruitment, division, and fusion of satellite cells adjacent to existing fibers (Alfei et al., 1994). These satellite cells provide a source of nuclei for both hypertrophy and hyperplasia events (Koumans and Akster, 1995).

Culture of muscle satellite cells have been developed for different fish species (Reviewed in Fauconneau and Paboeuf, 2000), but in vitro, these satellite cells only differentiated to very large myotubes for the studies undertaken on Oncorhynchus mykiss by some authors (Rescan et al., 1994; 19995;Faconneau and Paboeuf, 1998). We have recently observed that IGF system is implicated in proliferation and differentiation processes as well as in metabolical function in cultured Oncorhynchus mykiss satellite muscle cells like in mammals (Castillo et al., 2004). The accumulated evidence to date suggests that the major components of the IGF signalling system are also implicated in the growth of gilthead sea bream. During the past decade, in vivo studies on protein/energy ratio (Pérez-Sánchez et al., 1995; Martí-Palanca et al., 1996; Reviewed by Pérez-Sánchez an Le Bail, 1999) showed the nutritional regulation of the IGF-I axis in gilthead sea bream. In the same scenario, other works showed a positive correlation between IGF-I plasmatic values and changes in ration size in this species (Pérez-Sánchez et al., 1995; Company et al., 1999). Lately, it has been demonstrated the role of growth hormone (GH) and IGF-I on seasonal growth and the effect of diets with plant protein supply on nitrogen-metabolism and GH-liver axis (Mingarro et al., 2002; Gómez-Requeni et al., 2003, 2004).

Besides the growth actions of IGF-I, it has been demonstrated that both insulin and IGF-I, play a critical role in the regulation of metabolism. Several studies in fish reported the action of insulin and IGF-I on glucose and aminoacid uptake in distinct cell tissues (Inui et al., 1983; Negatu et al., 1995; Soengas and Moon, 1998; Gallardo et al., 2001; Castillo et al., 2004; Albalat et al. 2005). Moreover, Vega-Rubín et al., (2004) observed increases in glucose, glucagon and insulin plasma levels when arginin was injected intraperitoneally on gilthead sea bream, showing the interactions between aminoacid metabolism and glucose homeostasis in this specie. Recently, in our group, Rojas et al. (2005) partially cloned a specific glucose transporter (saGLUT4) in the red muscle of gilthead sea bream, describing the effects of different experimental diets on its expression. The same author reported that intraperitoneal administration of arginin on gilthead sea bream, provoked increases in the levels of GLUT4 protein in the white skeletal muscle.

All these data suggest an important role of IGFs in gilthead sea bream skeletal muscle, which remains to be analyzed in detail. Because the evidence that fish models can provide a complementary view to our understanding of the role of the IGF system, we use a primary culture of gilthead sea bream muscle cells previously described (Montserrat et al., 2006). The aim of this study is to analyze the role of IGFs on metabolic processes such as glucose and aminoacid uptake compared to insulin, as well as to identify the main signalling pathways undertaken by the IGF-I receptor by using specific inhibitors in gilthead sea bream muscle cells. Besides, we have detected the stimulation of GLUT4 in those cells in the presence of the different peptides throughout the cell culture.

2. Material and Methods

2.1. Chemicals

2-Deoxy-D-[2, 6-^{3}H] glucose (cat #TRK672), with a specific activity of 43 Ci/mmol and L-[2,3-^{3}H] alanine, with a specific activity of 52 Ci/mmol were purchased from Amersham Pharmacia Biotech Europe (Barcelona, Spain). Recombinant human IGF-I was purchased in from Peninsula Laboratories, Europe (Merseyside, UK). Other reagents were obtained from Sigma Aldrich Química (Alcobendas, Madrid). Anti-phospho-p44/42 (cat nº9106), 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).The remaining reactives were purchased from Sigma-Aldrich Química, S.A (Madrid, Spain). The okGLUT4 antibody was a kind gift from Dr. Josep Planas (University of Barcelona) and has been previously well characterized (Capilla et al., 2004).

2.2. Animals and cell culture

We used gilthead sea bream (*Sparus aurata*) with weights ranging from 2.8 to 8.5 g. Animals were provided by Aquamar S.L (San Fernando, Cádiz, Spain).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.

All experiments were conducted with cells seeded at a density of 2X 10^{6} per well in six-well plastic plates (9.6 cm2 /well, NUNC). Observations on morphology were regularly made to control the state of the cells, which were used at day4 (myocytes and recently differentiated myotubes) at day9 (mostly small myotubes) and day 11 (big myotubes) for 2-deoxyglucose (2-DG) and L-alanine uptake assays and Western blotting. All experiments were performed in triplicate; each condition was performed in triplicate (3 wells). Cells were incubated at 21°C, the optimal temperature for growth of the culture.

2.3. L-Alanine uptake assay

For L-alanine uptake assays, fish number and weight, as well as cell density were equivalent to that used for 2-DG uptake assays. After 4 or 10 days of culture, the culture medium (90% DMEM-FBS 10%) was aspirated and the cells were rinsed with ice-cold PBS and maintained in DMEM+0.5% BSA (DMEM-BSA) without FBS for 2–3 h. After preincubation with DMEM+0.5% containing different concentrations of peptides (from 10 to 100 nM for IGF-I and 100 nM to 1 μM for insulin) at different times (1 or 2 h), the medium was aspirated, rinsed two times with ice-cold PBS, and the cells were incubated with 1 μCi/ml of L-alanine for 20 minutes. Previously, time course experiments were performed to find the best conditions for the study (data not shown). The amino acid uptake was stopped by aspiration of the supernatant, followed by three rapid washes with ice-cold PBS. Next cells were solubilized with NaOH 0.1 N. Finally samples were placed in scintillation vials, and the radioactivity was counted (Packard Bioscience).

2.4. DG uptake assays

For 2-DG assays, 40–70 fish, with an approximate weight of 3, 5 g, were used for each culture. After pooling cells from all the animals of the same culture, the experiments were conducted with cells seeded at a density of $2X10^6$ per well in six-well plastic plates. The cells, after 4 or 9 days of culture, were incubated for 4 h with DMEM without FBS and after this period preincubated (30 or 60 min) in the presence or absence of insulin, IGF-I and IGF-II in DMEM-0.5% BSA (concentrations 100nM for IGF-I, IGF-II and 1μM for insulin). After preincubation, the cells were rinsed two times with ice-cold PBS and incubated with unlabeled 50 μM 2-DG together with labeled 2-DG (2 μCi/ml) in HEPES-saline buffer. The incubations with labeled and cold 2-DG, except for the time course experiments, were routinely of 30 min. The contents of the wells were aspirated and rinsed three times with ice-cold PBS, and the cells were lysed with NaOH 0.5 N. The contents of the wells were removed and placed into scintillation vials, and the radioactivity was quantified with a γ-counter (Packard Bioscience, Meriden, CT).

To better characterize glucose transport the effects of several compounds on glucose uptake stimulation by IGFs or insulin were analyzed at day 5 in the cell culture. PD-98059 is an inhibitor of the MEK1 protein, a component of the MAPK pathway; wortmannin is an inhibitor of the PI3K-Akt pathway; and cytochalasin B is a specific inhibitor of the facilitative glucose transporters. Cells were preincubated for 30 min

with wortmannin (1 μM) or PD-98059 (50μ M), and peptides (IGF-I, IGF-II or insulin) were added for 30 additional minutes. The cytochalasin B (20 μM) was added and incubated simultaneously with the labeled 2-DG for 30 min. Doses of inhibitors were selected from previous results obtained in similar experiments by Castillo et al. (2004).

2.5. Western blot analysis

In order to characterize the signal transduction pathways, following 5 days in culture, the cells were incubated with DMEM + 0.5 % BSA for 2-3 hours. Next cell were incubated in cells were preincubated for 30 min with wortmannin (1 μM) or PD-98059 (50 μM) Afterwards, cells 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 (at 1:500 dilution). 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).

Lysates of cells at day 4 and day 9, stimulated with 100 nM for IGF-I and IGF-II or 1μM for insulin for 1 hour were performed for the detection of GLUT 4. Inmunoblots were performed using the okGLUT4 antibody at 1:500 dilution in Tris-buffered saline containing 0, 1% Tween 20 and 5% non fat dry milk for 2 h at room temperature. The secondary antibody was used at 1:5000 dilution in the same buffer, and the detection of the luminescence was done using and enhanced chemi luminescence kit (Amersham).

2.6. Statistical analysis

The treatment was performed in triplicate for each experiment. Data are presented as means ± SE of at least three experiments. Statistical differences between conditions were analyzed by one-way analysis of variance (ANOVA) and the Tukey's test. Differences were considered statistically significant at $P< 0.05$.

3. Results

3.1. IGF-I, IGF-II and insulin effects on L-alanine uptake

The effects of IGF-I, IGF-II and insulin on alanine uptake were analyzed at day 4 in the cell culture. As shown in Figure 1, all the tested peptides stimulated alanine uptake over the basal values. IGF-I and IGF-II showed the highest stimulation of alanine uptake when comparing with insulin, considering that IGF-I and IGF-II concentration is 10 times lower than insulin.

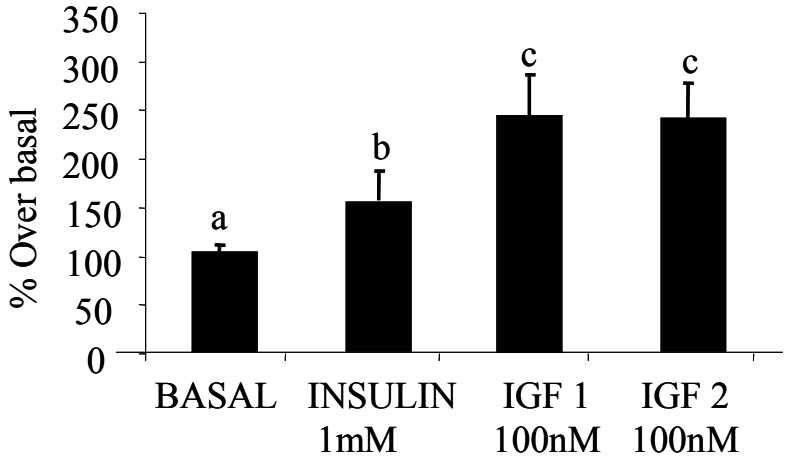

Figure 1. Stimulation of L-alanine uptake by insulin ,IGF-I and IGF-II . The cells, in *day 4* of in vitro development, were incubated for 2 h, and subsequently L-alanine uptake was quantified for 20 min. Results are expressed as percentage of stimulation over basal levels and mean SE (n =3 experiments). Different letters indicate significantly (P <0.05) different values among groups.

When the same experiment was carried out in cells at day 9 of the culture (myotubes), same tendency was observed, but the basal and stimulated alanine uptake was clearly lower than that found in myocytes of 4 days culture (data not shown).

3.2. IGFs and insulin effects on 2-DG uptake.

Figure 2 shows the effects of IGF-I, IGF-II compared with insulin on glucose uptake on muscle cells at day 4; preincubation with the peptides extended for 30 or 60 min and glucose uptake was fixed at 30 min. In general, preincubating the cells during 60 minutes in the presence of the different peptides resulted in higher stimulatory effects on glucose uptake. IGF-I and IGF-II showed the highest stimulation of glucose uptake when comparing with insulin effects.

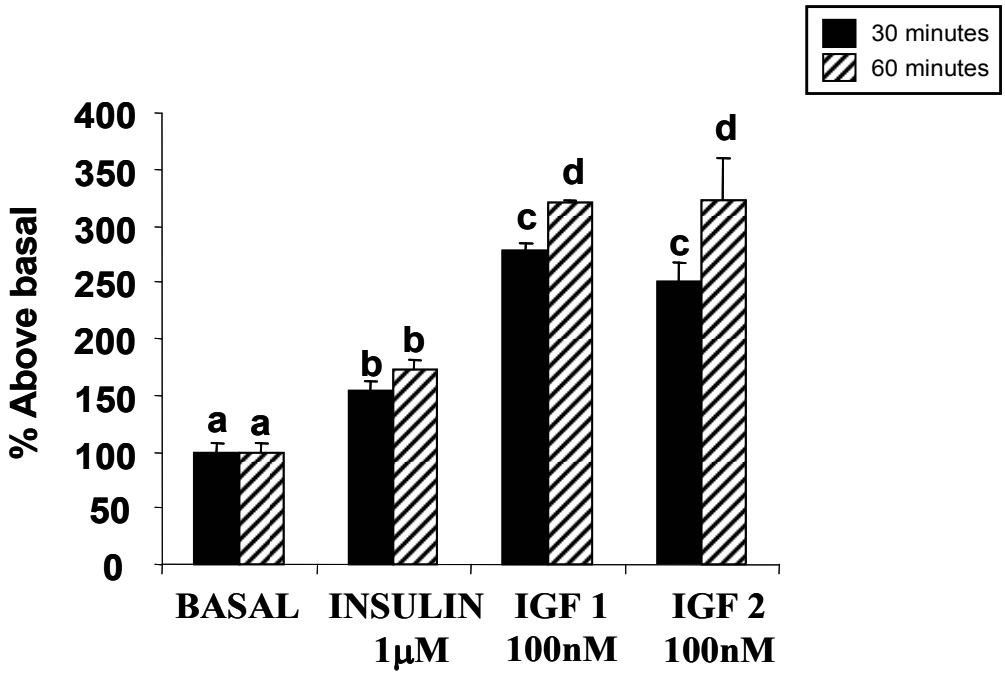

Figure 2. The 2-deoxyglucose uptake in muscle cells in culture stimulated by insulin, IGF-I or IGF-II. Cells were isolated and cultured for 4 days and incubated with the peptides (for 30 or 60 min) and subsequently incubated with 2 Ci/ml of labeled 2-deoxyglucose (2-DG) for 30 additional minutes. Results are expressed as percentage of stimulation over basal levels and means SE (n= 3 experiments). Different letters indicate significantly ($P < 0.05$) different values among groups.

As shown in Figure 3, same experiments were performed at day 9 in the cell culture and the stimulatory effects of both IGF-I and IGF-II were higher than those exerted by insulin after 30 or 60 minutes incubations.

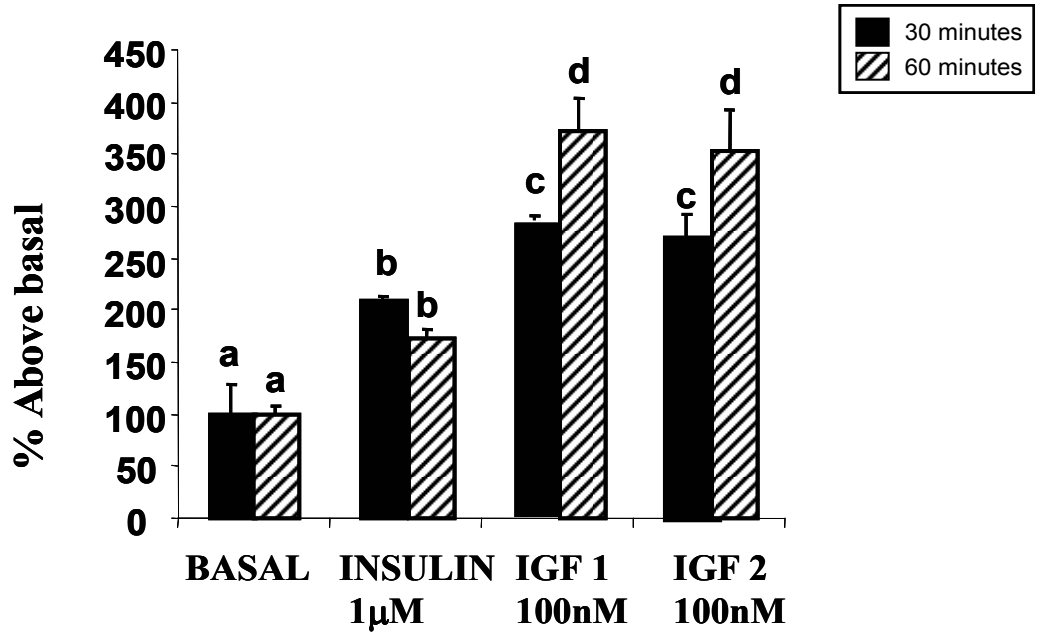

Figure 3. The 2-deoxyglucose uptake in muscle cells in culture stimulated by insulin, IGF-I or IGF-II. Cells were isolated and cultured for 9 days and incubated with the peptides (for 30 or 60 min) and subsequently incubated with 2 Ci/ml of labeled 2-deoxyglucose (2-DG) for 30 additional minutes. Results are expressed as percentage of stimulation over basal levels and means SE (n =3 experiments). Different letters indicate significantly ($P < 0.05$) different values among groups.

The effects of inhibitors of IGF-I, IGF-II and insulin-stimulated glucose uptake 5 day´s myocytes, are shown in figure 4. Both PD-98059 and wortmannin significantly inhibited the basal glucose uptake and the stimulatory effects of IGF-I, IGF-II and insulin. Same results were found when cells were incubated in presence of cytochalasin B.

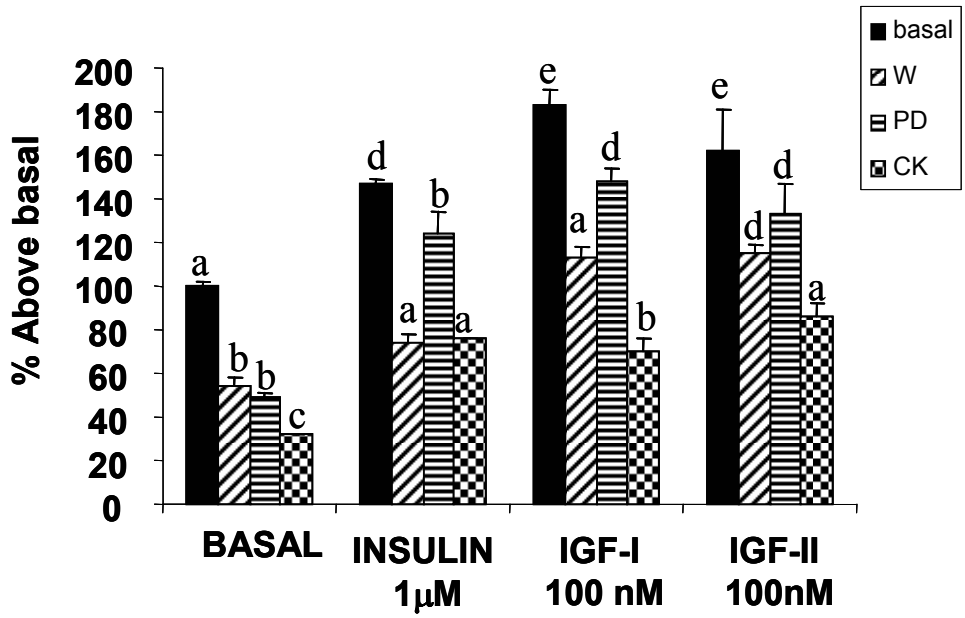

Figure 4.Effect of inhibitors on the 2-DG uptake in 5 day muscle gilthead sea bream cells. For wortmanin (Wort) (1 μM) and PD-98059 (PD) (50μ M), , the cells were preincubated for 30 min, and peptides [IGF-I, IGF-II or insulin (INS)] were added for 30 additional minutes. Cytochalasin B (CK) (20 μM) was incubated for the 30 min of 2-DG. Results are expressed as percentage of stimulation over basal levels and mean SE (n= 3 experiments). Different letters indicate significantly ($P < 0.05$) different values among groups.

3.3. Inhibition of Akt and MAPK signalling pathways

The effects of the specific inhibitors from both signalling pathways on day 5 cells are shown in table 1. Preincubation of cells with wortmannin (inhibitor of the Akt signalling pathway) reduced the phosphorylation of IGF-I and IGF-II-stimulated Akt protein (from 237% to 67% and 345% to 56% above basal values, respectively). The effect of PD-98059 (inhibitor of the MAPK signalling pathway) reduced the effects of both IGF-I and IGF-II on MAPK phosphorylation (from 146% to 34% and 231% to 45%, respectively).

	control	IGF-I 100nM	IGF-I 100nM +W (1mM)	IGF-I 100nM + PD 98059 (50mM)	IGF-II 100nM	IGF-II 100nM +W (1mM)	IGF-II 100nM + PD 98059 (50mM)
MAPK-P	100%^{a}	146%±23^{b}	123%±29^{a}	34%±4^{b}	231%±19^{a}	187% ±17^{a}	45%±9^{b}
Akt-P	100%^{a}	237%±17^{b}	67±17^{c}	212±17^{b}	345±17^{a}	56 %±17^{b}	314 %±17^{a}

Table 1 – Effect of inhibitors on the ERK 1/2 MAPK and PI3K- Akt pathways in IGF-I/IGF-II-stimulated 5 day muscle gilthead sea bream cells. 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 ($P<0.05$).

3.4. Immunodetection of GLUT4

With the use of an anti-okGLUT4 polyclonal antibody, a band of ~50 kDa was detected at 4 day and 9 day sea bream myoctyes. Specificity of the 50-kDa band was confirmed by the use of preimmune serum (data not shown). At day 4 the incubation of myoblast with 1μM insulin for 30 minutes resulted in GLUT4 stimulation (50% above basal levels); no changes of GLUT4 protein expression were observed when these cells were incubated neither IGF-I nor IGF-II (100nM). When myotubes of day 9 were incubated with 100nM IGF-I and IGF-II for 30 minutes, GLUT4 protein expression was increased ~200% above basal levels. The incubation with 1μM insulin did not provoke any stimulatory effect on GLUT4 protein levels at day 9.

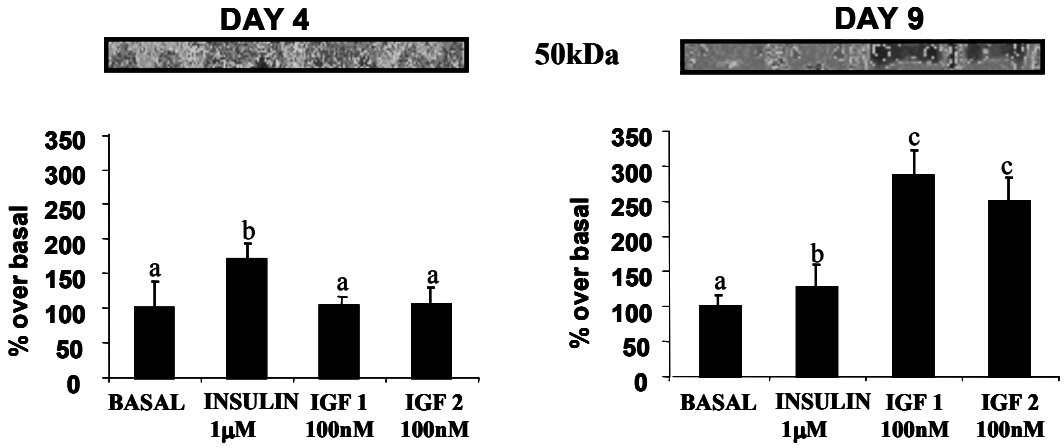

Figure 5. GLUT4 levels in gilthead sea bream muscle cells during development. Cells were incubated with insulin (1μM) or IGF-I or IGF-II (100 nM) for 60 minutes and lysed. 30 μg of protein were loaded in each lane and subjected to 10% SDS-PAGE under reducing conditions. Western blot was performed with primary antibody against GLUT4 (final dilution 1:500) and secondary antibody anti-rabbit (1:5000). Different letters indicate significant differences ($P<0.05$).

4.Discussion

This study is the first report assessing the metabolic effects of insulin and IGFs on cultured gilthead sea bream myocytes. Although the implication of insulin and IGF-I in growth processes have been reported in this species *in vivo*, the lack of information regarding the role of these peptides on sea bream muscle metabolism led us to examine the insulin and IGFs-stimulated uptake of metabolic substrates (2-DG and L-alanine). The use of specific inhibitors of the main signalling pathways confirmed that MAPK and PI3K pathways are implicated in glucose metabolism in gilthead sea bream cultured muscle cells. On the other hand, the inhibitory effect of cytochalasin B on insulin and IGFs -2DG stimulation, together with the detection of a GLUT4 transporter in sea bream myocytes, reveals the role of the studied peptides on sea bream glucose metabolism.

Muscle growth dynamics in fish results in large part from stimulation of protein synthesis which directly depends on amino acids availability (Brodeur et al., 2003). Our data clearly showed that both IGF-I and IGF-II are more effective than insulin in stimulating L-alanine uptake at day 4 in the cell culture. Previous work has demonstrated the stimulatory effects on amino acid uptake of insulin and IGF-I, respectively, in fish muscle (Inui et al., 1983; Negatu et al., 1995). In the same manner, studies on stable cell lines (Benguinot et al., 1985) and in human or rodent muscle strips (Bevan et al., 1992), demonstrated the stimulatory effects of IGF-II in L-alanine uptake.

More recently, higher stimulatory effects for IGF-I than for insulin have been demonstrated in trout isolated cardiomyocytes (Gallardo et al., 2001) and on cultured rainbow trout myocytes (Castillo et al., 2004). However, the stimulatory effect of the peptides on L-alanine uptake, is more effective in previous stages of the cell culture (day 4) than at day 10 (data not shown). This data suggest that amino acid requirement seems to be more important during proliferative stages than during cell muscle development (Castillo et al., 2004).

Fish are generally thought to have a limited ability to utilize carbohydrate when compared with mammals (Legate et al., 2001) and the skeletal muscle is the main tissue responsible for eliminating plasma glucose in brown trout after a glucose load (Blasco et al., 1996). In the current study, we investigated the role of IGF-I and IGF-II compared with insulin, in the 2-DG uptake in sea bream cultured myocytes.

The present data showed that IGFs are more effective than insulin in glucose uptake stimulation in both myoblast and large myotubes. However, the stimulation increased as well as cells differentiated in the cell culture. Previous studies in cultured rainbow trout myocytes showed similar results (Castillo et al., 2004), but no data is available in gilthead sea bream muscle. Recently, Albalat (2005) demonstrated that IGF-I had higher stimulatory effects in the 2-DG uptake than insulin on isolated gilthead sea bream adipocytes. In the same way, previous work in mammals showed that IGF-I stimulated 2DG-uptake (Beguinot et al., 1985; Wang et al., 1987; Niu et al., 2003) and IGF-II resulted a positive stimulator of 2-DG in rat and human muscle strips (Yu and Czech 1984; Bervan et al., 1992; Zierath et al., 1992). Although type II IGF-receptor have been identified in our group by Méndez et al. (2001) in brown trout at different stages of the development, studies in mammalian cells based on ligand competitions (Ewton et al., 1987), IGF-II analogues (Burguera et al., 1994) and the use of antibody to the type II IGF-receptor (Kiess et al., 1987), suggested that the biological effects of both IGFs are mediated by the type I IGF-receptor.

The results in this study demonstrated that both IGFs had higher stimulatory effects than insulin on the stimulation of 2-DG glucose uptake, and in concentration similar to these described by Castillo et al. (2004) in rainbow trout myocytes. Increases in absolute 2-DG uptake stimulation by IGFs throughout the cell culture might be related with the reported increase in the IGF-I receptor levels as well as with the higher metabolic needs in differentiated myotubes (Montserrat et al., 2006).

In order to determine the specificity underlying the glucose uptake process, we incubated 4 day-sea bream myocytes in the presence of PD-98059 and wortmannin. In agreement with the observations of Castillo et al. (2004) in rainbow trout myocytes, we found out a decrease on insulin and IGFs- 2-DG stimulation, which indicates that MAPK and PI3K pathways are implicated in glucose metabolism in fish muscle. In addition, the use of cytochalasin B blocked the stimulatory effects of insulin and IGFs, suggesting that glucose transport takes place thought specific facilitative transporters in sea bream cultured myocytes. Previous studies on fish reported the existence of cytochalasin B-sensitive glucose transport in red blood cells of the eel (Tse and Young, 1990; Soengas and Moon, 1995) the primitive hagfish (Young et al., 1994) and in the black bullhead (Soengas and Moon, 1998). More recently, 2-DG uptake was inhibited by cytochalasin B in Xenopus laevis oocytes expressing an insulin-responsive glucose transporter (GLUT4) cloned from Onchorynchus kisutch (**ok**GLUT4) (Capilla et al. 2004). These previous findings led us check the presence of the specific GLUT4 transporter in sea bream myocytes. In the present study, GLUT 4 protein synthesis is stimulated by IGFs and insulin in a different manner throughout the cell culture. These data suggest that insulin and IGFs stimulate GLUT4 protein synthesis. In mammals, several studies reported the stimulation of IGF-II in GLUT4 protein synthesis (Kaliman et al., 1998), as well as the effects of insulin on GLUT4 transcription in human muscle cell cultures (Al-Khalili et al., 2003). Current efforts in our laboratory are underway to examine whether insulin and IGFs stimulate the protein synthesis of GLUT4 in gilthead sea bream muscle cells.

On the other hand, it has been widely described in mammals that acute insulin treatment stimulates glucose transport in adypocytes and myocytes, largely by mediating translocation of GLUT4 from intracellular compartment to the plasma membrane (Pessin et al., 2000). In the same way, the effects of IGF-I in the 2-DG glucose uptake on L6 cells over expressing GLUT4 transporter where higher than in wild type L6 cells (Lawrence et al., 1992).

Previous findings in our group showed the stimulatory effects of insulin and IGFs on Akt phosphorylation in cultured sea bream myocytes (Montserrat et al., 2006). The present work suggested that such an increase in Akt phosphorylation throughout the cell culture could be related of the observed increase of GLUT4 protein content after insulin and IGFs incubations, and this observation is also supported by wortmannin and cytochalasin B results. In fact, studies in a number of cell types including myoblast,

myotubes and adipocytes, related GLUT4 translocation to the plasma membrane with PI3K promotion of Akt phosphorylation (Wilson et al., 1995; Zorzano et al., 2000; Saltiel et al., 2001; Rauch et al., 2005). In the same way, it has been shown that expression of either constitutively active PI-3K (Tengholm et al., 2002) or Akt (Kohn et al., 1996) is sufficient to stimulate GLUT4 translocation and insertion to the plasma membrane to an extent comparable to that observed with insulin alone. Thus, the increase in the insulin-IGFs-stimulated increment in 2-DG uptake in sea bream myocytes, may be in part explained by either enhanced insulin-IGFs-stimulated -GLUT4 protein synthesis, or translocation to the transporter to the membrane through PI3-kinase-Akt phosphorylation reported in this study.

In conclusion, stimulatory effects of both IGF-I and IGF-II on L-alanine uptake where higher than those found by insulin in 4 days sea bream myosatellite cells, and this stimulatory effect decreased with cell differentiation. In the same way, again both IGFs exerted higher stimulatory effects than insulin in 2-DG uptake, being more remarkable at day 9 myotubes. The stimulatory effects from both IGF-I and IGF-II in 2-DG uptake in differentiated myotubes, together with the stimulation of GLUT4 protein content, reveals the important role of those peptides in glucose fish metabolism. Interestingly, these findings showed as a first time in this specie the metabolic functions of IGF-II in sea bream myosatellite cells. Thus, our *in vitro* sea bream myocytes culture system offers a suitable model to further analyze the link between nutritional status and skeletal muscle function in fish.

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