Insulin and insulin-like growth factor I signaling pathways in rainbow trout (Oncorhynchus mykiss) during adipogenesis and their implication in glucose uptake

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Insulin and insulin-like growth factor I signaling pathways in rainbow trout (Oncorhynchus mykiss) during adipogenesis and their implication in glucose uptake. Am J Physiol Regul Integr Comp Physiol 299: R33–R41, 2010. First published March 17, 2010; doi:10.1152/ajpregu.00457.2009.—Primary cultures of rainbow trout (Oncorhynchus mykiss) adipocytes were used to examine the main signaling pathways of insulin and insulin-like growth factor I (IGF-I) during adipogenesis. We first determined the presence of IGF-I receptors (IGF-IR) and insulin receptors (IR) in trout preadipocytes (day 5) and adipocytes (day 14). IGF-IRs were more abundant and appeared to be in higher levels in differentiated cells than in preadipocytes, whereas IRs were detected in lower but constant levels throughout the culture. The cells were immunoreactive against ERK1/2 MAPK, and Akt/Pi3K, components of the two main signal transduction pathways for insulin and IGF-I receptors. Stimulation of MAPK phosphorylation by IGF-I was higher in preadipocytes than in adipocytes, while no effects were observed in MAPK phosphorylation after incubation of cells with insulin. Akt phosphorylation increased in the presence of both insulin and IGF-I, with higher levels of stimulation in adipocytes than in preadipocytes. Activation of both pathways was blocked by the use of specific inhibitors of MAPK (PD98059) and Akt (wortmannin). We describe here, for the first time, the effects of IGF-I and insulin on 2-deoxyglucose uptake in primary culture of trout adipocytes. IGF-I was more potent in stimulating glucose uptake than insulin, and PD98059 and wortmannin inhibited the stimulation of glucose uptake by this growth factor, suggesting that IGF-I plays an important metabolic role in trout adipocytes. Our results suggest that differential activation of the MAPK and AKT pathways are involved in the IGF-I- and insulin-induced effects of trout adipocytes during the various stages of adipogenesis. fish adipocytes; insulin and IGF-I receptors; MAPK and AKT; glucose uptake; GLUT4

Vertebrate adipose tissue plays a critical role in energy balance; it either accumulates lipids in the form of triglycerides or breaks down these lipid stores into energy fuel supply. In fish, adipose tissue is located periviscerally and, as in mammals, its mass changes with nutritional status (18, 34). The use of high-lipid feed for cultured fish can lead to excessive lipid deposition, a situation that, especially in salmonids, can resemble human obesity. Adipose tissue is also considered an endocrine organ, capable of secreting many substances with endocrine, paracrine, or autocrine actions (6, 22, 31, 62). Adipose tissue mass can expand notably during a lifetime by an increase in the volume of mature cells but also by a process of proliferation and differentiation of adipocyte precursor cells found in the stromal vascular fraction of adipose tissue. Studies with primary cell culture of adipocytes have helped to understand the complexity of adipogenesis, lipid accumulation, and their regulation (6, 27, 53). Recently, we developed the culture conditions to differentiate adipocytes from their precursor cells obtained from rainbow trout (Oncorhynchus mykiss) adipose tissue (7). This culture system will allow us to study adipogenesis in fish, as well as its endocrine regulation and metabolism.

In mammals, insulin and insulin-like growth factor I (IGF-I) play an important role in the regulation of many physiological processes, and adipose tissue is one of the relevant target tissues of these peptides. Circulating IGF-I is mainly produced by the liver, but IGF-I is also produced locally and ubiquitously among the vertebrates studied (3). IGF-I can stimulate both mitogenesis and differentiation in various types of cells, including adipocytes (3, 36). Insulin is mainly involved in the regulation of glucose and lipid metabolism, but it also controls growth and differentiation in adipose tissue (33). In fish, the insulin and IGF-I effects on adipose tissue are not well known, but the relative importance of their effects on fish muscle appears to be different to that in mammals; IGF-I acts as a growth factor, but it is also very important in the control of piscine muscle metabolism (10, 11).

The biological action of both peptides is mediated through their receptors, IGF-I receptor (IGF-IR) and insulin receptor (IR), members of the tyrosine kinase family. IGF-IR and IR are similar heterodimeric proteins composed of two extracellular α-subunits containing the ligand-binding site and two β-subunits containing the tyrosine kinase domain (21, 35). In fish, the presence of functional IGF-I and insulin receptors has been detected in a variety of fish tissues (37, 46, 47, 48, 50), and a high degree of sequence similarity with mammalian receptors has been observed (23, 24). Contrary to mammals, IGF-I receptors are more abundant than insulin receptors in all fish tissues analyzed (42, 46, 47). The binding properties of these receptors have been well studied in fish tissue homogenates, including brown trout (Salmo trutta) adipose tissue (48) and in primary cell culture of trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) myocytes (11, 39). IGF-I and insulin binding induces autophosphorylation and activation of the receptor tyrosine kinase activity, leading to the activation of two major intracellular signaling pathways, the MAPK and the phosphatidylinositol 3-kinase (PI3K/AKT), which, in turn, regulate several transcriptional events involved in mitogenesis, proliferation, and differentiation. These two pathways have been well studied in mammalian adipose tissue (33) and cell lines like the 3T3-L1 adipocytes (15, 26). Studies using 3T3-L1 preadipocytes have shown that IGF-I stimulates pro-
liferation through activation of the MAPKs pathway (3) and that this stimulation of MAPK by IGF-I decreases during the early stages of differentiation (4, 5), whereas IGF-I activation of the PI3K/AKT pathway stimulates adipogenesis, provoking the differentiation of 3T3-L1 preadipocytes (15, 63).

As far as we know, the mechanisms mediating IGF-I and insulin effects are not known in fish adipose tissue. Nevertheless, several studies have elucidated the effect of IGF-I and insulin as ligands on the activation of MAPK and AKT signaling pathways in the embryonic zebrafish ZF-4 cell line, and in rainbow trout and gilthead sea bream muscle cells (11, 17, 39, 50). These studies revealed that activated MAPK activity level was high in proliferating myocytes but decreased during the differentiation stage, while PI3K/AKT phosphorylation increased in differentiated myocytes.

In contrast to mammals, fish are known to be intolerant to glucose (28, 40), but the role of adipose tissue in glucose utilization is not well understood. Insulin and IGF-I stimulated glucose uptake in fish skeletal muscle cells (11, 17, 20). Furthermore, the stimulation of glucose transport by insulin in freshly isolated rainbow trout adipocytes has been reported (9), probably by the translocation of GLUT-4 vesicles to the plasma membrane.

The signaling mechanisms by which insulin and IGF-I act on the transport of glucose are still not completely clear in fish. Most studies in mammals consider that the insulin-induced glucose uptake is mediated by AKT pathway activation, although the MAPK pathway may also contribute to this process (13, 29, 32, 64). The aim of this study was to identify the presence of IGF-IR and IR throughout the culture evolution (13, 29, 32, 64). The aim of this study was to identify the presence of IGF-IR and IR throughout the culture evolution and to elucidate the effect of insulin and IGF-I on the signal transduction pathways in our trout adipocyte culture system. We have analyzed the insulin and IGF-I effects on the phosphorylation of MAPK and AKT signaling pathways in trout preadipocytes and adipocytes in culture and their relation to the activation of glucose uptake by those peptides.

MATERIALS AND METHODS

Cell culture. Rainbow trout (O. mykiss) were supplied by “Truites del Segre” fish farm (Lleida, Spain) and maintained in the “Servei d’Estabulari de la Facultat de Biologia” (Universitat de Barcelona, Spain). All animal-handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona (procedure no. 4002), following the EU-Spanish and Catalan Government established norms and procedures.

Adipose tissue was obtained following the protocol described previously by Bourauel et al. (7) with some modifications. Animals weighing 200–250 g were dissected under sterile conditions; fragments of perivisceral adipose tissue were collected in Krebs-HEPES buffer (pH 7.4) and were digested for 1 h with collagenase 130 U/ml (type II; Sigma-Aldrich, Madrid, Spain) at 18°C. After filtration, cell suspension was centrifuged at 700 g for 10 min at room temperature. The cell pellet was then subjected to a second centrifugation at 700 g for 10 min at room temperature. The medium was aspirated, and cells were washed with ice-cold PBS (10 mM NaCl, 10 mM KHCO3, and 0.1 mM EDTA) for 10 min at room temperature. The cell pellet was then resuspended in Leibovitz L-15 medium containing 10% FBS, 2 mM l-glutamine and antibiotics (growth medium). Cells were counted, diluted, and plated into flasks (25 cm2) at a concentration of 4 x 104 cells per cm2 at 18°C. After confluence (day 7), cells were put into a “differentiation medium” containing growth medium supplemented with 10 μg/ml insulin, 0.5 mM IBMX, 0.25 μM dexamethasone, and lipid mixture (10 μM/ml) (Sigma-Aldrich). Media were changed every 2 days during the whole procedure. Until day 7, the growth medium was used, and after day 7, cells were maintained in differentiation medium. For the different experiments, the cells were used at two different time points of development determined by observation under the microscope and according to Bourauel et al. (7). Counting from the plating day (day 0), the cells were studied at a preadipocyte state (day 5) and at a fully differentiated state (which was day 11 or day 14 depending on the culture).

Immunofluorescence. To detect the presence of the IGF-IR, cells were cultured on glass cover slips treated previously with 1% gelatin until day 5 (preadipocytes) and 14 (adipocytes). Briefly, cells were fixed with paraformaldehyde for 15 min and permeabilized with 0.05% Triton X-100 for 10 min at room temperature. After washing, cells were blocked with 1% BSA in PBS-glycine (blocking buffer) for 20 min and incubated with primary antibody against IGF-IR β-subunit (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in blocking buffer. Simultaneously, control cells were treated with blocking buffer alone. After 1 h, all of the cells were incubated with a secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit (1:500), in combination with a Hoechst stain (1:1,000). Cells were mounted with Mowiol after a wash with PBS, and immunofluorescence was captured with a confocal microscope (Confocal Olympus Fluoview 500).

Cell incubation and AKT and MAPK Western blot analysis. Cells at day 5 (preadipocytes) and day 11 (adipocytes) were used to study the intracellular signal transduction pathways of IGF-I and insulin and the effect of their inhibitors. At the beginning of the experiment, cells from these days were serum starved in Leibovitz L-15 medium, containing only 0.5% of FBS for 4–6 h. Then, they were incubated with the same media in the presence of recombinant human IGF-I (100 nM, Bachem, UK) or porcine insulin (1,000 nM, Sigma, Spain) for 30 min at 18°C. The validation for the use of these heterologous peptides for fish experiments has been reported previously using a primary culture of trout muscle cells (11, 17). Simultaneously, another set of cells was treated with PD98059 (50 μM) or wortmannin (1 μM), inhibitors of the MAPK and PI3K/AKT pathways, respectively, for 30 min and IGF-I or insulin were added for 30 additional minutes. Cells incubated without peptides or inhibitors represented the control. The medium was aspirated, and cells were washed with ice-cold PBS and lysed with lysis buffer (10 mM Tris·HCl, 140 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Nonidet P-40, H. 7.6). The lysates were centrifuged at 13,000 g for 15 min at 4°C, the supernatants were recovered, and the protein was measured (8).

For Western blots, equal amounts of protein (50 μg) were run on SDS/polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane for 2 h at 100 V. After 3 x 5 min washes with washing buffer (10 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.5), the membrane was blocked with washing buffer containing 5% dry milk for 2 h at 18°C. The immunoblotting was performed using primary antibodies (Cell Signaling Technology, Denver, CO) against total MAPK (MAPK-T) and AKT (AKT-T) proteins and their phosphorylated forms MAPK-P, AKT-P (1:500), followed by incubation with the secondary antibodies (1/5,000, Santa Cruz Biotechnology) for MAPK (anti-mouse) and AKT (anti-rabbit). Bands were developed with enhanced chemiluminescence.

We also identified the presence of IGF-I receptors (IGF-IR) and insulin receptors (IR) in preadipocyte (day 5) and adipocyte (day 14) cells, using primary antibodies against the human IGF-IR and IR β-subunit (1:500; Cell Signaling Technology). The C-terminal region, against which the antibodies used were raised, corresponds to the tyrosine kinase domain, a very conserved region between fish and mammals (81–88% between human and trout) (23, 24). An anti-rabbit was used as a secondary antibody (1:5,000). Homogenates of perivisceral adipose tissue from rats were used as a positive control. Bands were visualized with enhanced chemiluminescence.
Glucose uptake assay. In adipocyte differentiated cells (day 11), 2-deoxyglucose (2-DG) uptake was measured stimulated by IGF-I and insulin. Briefly, cells were cultured in 24-well plates at a density of 4 × 10^4 cells/cm². At day 7, cells were induced to differentiate in a differentiation medium as described above. At day 11, differentiated cells were incubated with two different concentrations for each peptide according to previous studies (9, 15). Cells were serum starved for 4–6 h and pretreated without (basal) or with IGF-I (10 and 100 nM) or insulin (100 and 1,000 nM) in Leibovitz L-15 medium containing 0.5% BSA for 30 min. After two washes with ice-cold PBS, cells were incubated with HEPES-buffered saline containing labeled 2-DG (2 μCi/ml) or unlabeled 50 μM 2-DG (transport solution) for 30 min at 18°C. The transport solution was then aspirated, and cells were washed with ice-cold PBS containing 50 mM glucose. Finally, cells were lysed with NaOH 0.5 N, and radioactivity was determined by scintillation counting. Protein concentration was measured following the method by Bradford (8).

To understand the different mechanisms of IGF-I and insulin on signal transduction involved in glucose uptake, the effect of specific inhibitors was studied. Cells were treated with a MAPK inhibitor (PD98059, 50 μM) or an AKT inhibitor (wortmannin, 1 μM) for 30 min and IGF-I (100 nM) or insulin (1,000 nM) were added for 30 additional minutes. Cytochalasin B (20 μM), a glucose transporter inhibitor, was added and incubated simultaneously with the labeled 2-DG for 30 min. The glucose uptake assay was performed as described above.

Statistical analysis. Data are presented as means ± SE. Results were analyzed by one-way ANOVA, followed by Duncan’s test, using SPSS 11.5 for Windows. Differences were considered statistically significant at P < 0.05.

RESULTS

Immunodetection of IGF-IR and IR. First, we investigated the presence of IGF-IR and IR in trout preadipocyte (day 5 of culture) and adipocyte (day 14) cells. Western blot analysis revealed the presence of both IGF-IR and IR in cultured trout cells (Fig. 1). The intensity of the reactive band for IGF-IR antibody was clearly higher in mature adipocyte cells than in preadipocytes (Fig. 1, A and C). The presence of IR was similar at both stages of culture, and apparently lower with respect to IGF-IR at day 14 (Fig. 1, B and D). Immunofluorescence of fixed cells revealed the presence of IGF-IR at both stages of cell culture with a higher signal at day 14 (Fig. 2). The receptor was clearly present at the plasma membrane, as well as in the nuclei and scattered throughout the cytoplasm.

Activation of MAPK and AKT pathways by IGF-I. The total and activated forms of MAPK and AKT (MAPK-T, AKT-T, MAPK-P, and AKT-P) were analyzed in lysates from cultured proliferated preadipocyte cells (day 5 of culture) and differentiated adipocyte cells (day 14) in response to IGF-I (Fig. 3). The addition of IGF-I (100 nM) induced an increase in MAPK-P levels in both preadipocytes and adipocytes (Fig. 3A). The level of activation was significantly higher than in control cells (incubated without hormone) in preadipocytes treated with the peptide (163.96% ± 37.09% over control), while the increased MAPK-P levels in IGF-I-incubated differentiated adipocytes was not significantly different with respect to control adipocytes (144.68% ± 17.50%). Preincubation of cells with a specific MAPK inhibitor, PD98059, for 30 min, revealed a decrease in MAPK-P in preadipocytes (85.29% ± 7.47%) and adipocytes (47.88% ± 11.80%). Moreover, we observed that total MAPK protein expression was unaltered by the IGF-I treatment in preadipocytes and adipocytes (data not shown).

As shown in Fig. 3B, the exposure of preadipocytes and adipocytes to IGF-I (100 nM) for 30 min resulted in an increase in the AKT phosphorylated form. Contrary to MAPK activation, the magnitude of this effect was significantly different in adipocytes (188.35% ± 31.63%) but not in preadipocytes.
(152.75% ± 47.25) compared with cells in the absence of hormone. The treatment of cells with wortmannin, a specific inhibitor of the AKT pathway, for 30 min resulted in a significant decrease of AKT phosphorylation in preadipocytes (14.22% ± 0.10) and adipocytes (8.60% ± 4.57). No effect of IGF-I was observed on total AKT in both preadipocytes and adipocytes (data not shown).

Activation of MAPK and AKT pathways by insulin. Incubation of cells with insulin (1,000 nM) for 30 min revealed no effect of this hormone on MAPK phosphorylation in either preadipocytes or adipocytes (130.46% ± 47.07, 84.65% ± 13.48, respectively) (Fig. 4A). The effect of PD98059 was not significant in preadipocytes (61.81% ± 22.25), but the level of activation significantly decreased in adipocytes (53.26% ± 16.21) compared with the control cells. As shown in Fig. 4B, exposure of cells to insulin (1,000 nM) for 30 min resulted in a significant increase of AKT phosphorylation only in adipocytes (163.87% ± 21.77) but not in preadipocytes. The use of wortmannin significantly inhibited AKT phosphorylation over control in both preadipocytes (13.34% ± 12.89) and mature adipocytes (39.79% ± 30.22). MAPK and AKT total levels revealed no changes in preadipocytes and adipocytes, neither with the stage of culture or after insulin administration (data not shown).

IGF-I and insulin effects on 2-deoxyglucose uptake. Next, we investigated the effects of two concentrations each of IGF-I (10, 100 nM) and insulin (100, 1,000 nM) on the transport of 2-DG in adipocytes (day 11) (Fig. 5). IGF-I showed dose-dependent stimulation of glucose uptake that was significantly different at 100 nM. On the contrary, a very slight stimulation by insulin was observed at 1,000 nM. Cytochalasin B was used as a specific inhibitor of glucose transport, which provoked a significant inhibition of glucose uptake, reaching a value of 6% over basal uptake.

![Fig. 2. Immunolocalization of IGF-IR in preadipocytes (B) and adipocytes (C). Cells were fixed and probed with primary IGF-IR β-subunit antibody (1:50) and a secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit (1:500). Cell nuclei were counterstained with Hoechst staining in blue. Control cells were incubated with blocking buffer alone (A). Scale bar = 10 μm. The white arrows indicate the plasma membrane localization.](http://ajpregu.physiology.org/)

![Fig. 3. Phosphorylation of MAPK and AKT (MAPK-P, AKT-P) by IGF-I in trout preadipocytes and adipocytes. Cells were incubated without (control) or with IGF-I (100 nM) for 30 min. Also, cells were incubated with MAPK inhibitor (PD98059, 50 μM) or AKT inhibitor (wortmannin, 1 μM) for 30 min; then IGF-I was added for 30 additional minutes. Cell lysates were prepared and immunoblotted with MAPK antibody (A) or AKT antibody (B), as described in the MATERIALS AND METHODS. Densitometry analysis of three independent experiments (*n* = 3 for each experiment) was normalized against the content of total MAPK or AKT. Results are expressed as variation over control values (100%). *a,b,c* Different letters indicate significant differences (*P* < 0.05).](http://ajpregu.physiology.org/)
In a second experiment, the effect of the inhibitors wortmannin and PD98095 on 2-DG uptake was studied. Wortmannin reduced basal 2-DG uptake slightly, but PD98095 caused a significant decrease (Fig. 6). In addition, both inhibitors significantly reduced IGF-I stimulation of glucose uptake in adipocytes, whereas these inhibitors only slightly reduced the glucose uptake in cells treated with insulin. Cytochalasin B clearly inhibited the transport of glucose in adipocytes in all conditions.

**DISCUSSION**

**Identification of IGF-I and insulin receptors in trout preadipocytes and adipocytes.** This is the first study in which IR and IGF-IR receptors are analyzed in cultured fish adipocytes. The presence of both types of receptors in these cells has been demonstrated by Western blot in preadipocytes and mature adipocytes. Also, by immunofluorescence, the IGF-IR has been shown to be localized, as in mammalian cells, at the plasma membrane, in the cytoplasm and in the nuclei (14, 51). Our results are in agreement with studies of IGF-I and insulin binding in cultured muscle cells from trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) (12, 39). These studies have shown that IGF-I receptors, more abundant and with higher affinity than insulin receptors, increased in number as cell differentiation progressed, similar to what we have observed in our cell culture. Immunoblotting and binding studies in embryonic zebrafish cells have revealed the presence of IGF-I receptors (50), although insulin receptors were not analyzed. In fish, but not in mammals, the same IGF-IR preponderance over insulin receptors is also found in a variety of adult tissue homogenates, including adipose tissue (37, 46, 47, 48).

IR and IGF-IR are expressed on the surface of mammalian adipose cells in culture, and their relative presence changes depending on the various models and the timing of the culture. Nevertheless, the relative proportion of both receptors and their expression throughout the culture is different from what has been described in fish cells (21, 42). In this sense, in 3T3-L1 and brown preadipocyte cell lines, the IGF-IRs were abundant and constant in undifferentiated and differentiated cells, whereas IRRs were low in undifferentiated cells and increased with differentiation (21, 38, 55).

IGF-I is considered to be the major regulator of preadipocyte cell lines and primary cultures that induces both mitogenic and
differentiation responses in vertebrates (52, 53, 54, 57). The presence of IGF-I receptors in trout preadipocytes and differentiated cells suggests a role for IGF-I in adipocyte proliferation and differentiation. In agreement with these data, we have previously demonstrated that IGF-I stimulates the proliferation of trout preadipocytes (7). This stimulation has also been observed in mammalian cells and is mediated through the IGF-I receptor (3). IGF-I may also be involved in the differentiation of fish adipocytes, as it is in mammals, but this has still to be demonstrated.

Insulin is required for adipocyte differentiation in mammals and birds (25, 52). As in the case of IGF-I, there is scarce information about the effect of insulin on cell adipocytes in fish. Oku et al. (45) have described the stimulatory effect of insulin (5 \mu M) or cytochalasin B (Cch; 20 \mu M) for 30 min before the incubation with IGF-I (100 nM) or insulin (INS; 1,000 nM) for an additional 30 min. Results are expressed as a percentage of stimulation over basal levels (100%) and mean ± SE (n = 2 experiments). 

The analysis of MAPK activity in the present study indicated that stimulation of MAPK-P by IGF-I is higher in preadipocytes than in adipocytes, which suggests a probable relationship with the proliferation process.

In contrast to the positive effect of IGF-I on phosphorylation of MAPK, insulin had no effect on this pathway during the adipocyte culture, suggesting that this insulin signaling pathway may not be functional in these cells.

Several studies in other fish cell types, such as the embryonic zebrafish ZF-4 cell line, have shown the stimulatory effect of IGF-I and IGF-II on the MAPK pathway during proliferation and early differentiation (50). IGF-I and insulin also activated MAPK-P in cultured rainbow trout and gilthead seabream muscle cells (11, 17, 39), especially at the proliferation stage, in agreement with the results obtained in trout adipocytes. Nevertheless, to better understand these processes, further studies should be performed, for example, analyzing downstream molecules of the signaling pathways, like the transcription factors forkhead box O, peroxisome proliferator activated receptors and cAMP response element-binding.

**Effect of IGF-I and insulin on MAPK and AKT pathways.** In mammals, the MAPK and PI3K/AKT pathways are the main signaling transduction pathways activated by IGF-I and insulin. IGF-I and insulin stimulate proliferation of 3T3-L1 preadipocytes and fetal brown adipocytes through MAPKs (1, 4). This activation of MAPK decreases with differentiation of 3T3-L1 cells (4). In contrast, the AKT pathway is more involved in adipogenic differentiation; for instance, in 3T3-F442A cells, IGF-I and insulin regulate differentiation mediated by PI3K/AKT activation (15). The process of differentiation is accompanied by the expression of genes and enzymes, such as those involved in fatty acid synthesis and lipid accumulation (25). PD98059 and wortmannin, effective inhibitors of MAPK and AKT, respectively, have confirmed the contribution of these pathways in cell proliferation and cell differentiation (49, 56, 60).

We previously characterized the proliferation and differentiation process in trout adipocytes (7). In that study, we demonstrated that cell proliferation is maximal in preadipocytes of between 5 and 7 days of culture and that IGF-I stimulates the proliferative capacity at the preadipocyte stage. The analysis of MAPK activity in the present study indicated that stimulation of MAPK-P by IGF-I is higher in preadipocytes than in adipocytes, which suggests a probable relationship with the proliferation process.
in rainbow trout and gilthead sea bream muscle cells, when incubated with insulin and IGF-I (11, 17, 39).

Therefore, taking into account all of the data mentioned above, activation of both AKT and MAPK pathways by insulin and IGF-I appears to be similar to the patterns of activation in mammals, in the sense that these peptides activate different intracellular signal transduction pathways depending on the stage of the cell culture development in trout adipocytes and myocytes. However, a specific trend in piscine cells seems to be that these signaling pathways are activated more efficiently by IGF-I than by insulin, as previously observed in trout myocytes, and in agreement to the lower ratio of IR/IGF-IR described in fish compared with mammalian tissues (10, 11).

**Stimulation of glucose uptake by IGF-I and insulin in trout adipocytes.** Our study is the first one to describe the effects of IGF-I and insulin on 2-DG uptake in primary culture of trout adipocyte cells. We have previously reported that insulin was able to stimulate glucose uptake in freshly isolated trout adipocytes (9). Furthermore, the 2-DG uptake assay technique has been previously used to test the effects of hormones on fish myocytes (10, 17). The fact that IGF-I was more potent in stimulating glucose uptake than insulin, suggests that IGF-I plays an important metabolic role in fish adipocytes, in agreement with the high efficiency of IGF-I stimulating the signaling pathways and with the finding that IGF-I receptors seem to be more abundant in trout adipocyte cells than those of insulin.

The same difference in potency for both peptides was reported in rainbow trout myocytes (10), where IGF-I was more efficient at stimulating glucose transport than insulin. Recently, the effect of IGF-I and IGF-II on glucose metabolism has been studied in trout myocytes in culture, with IGF-I and IGF-II also being more potent than insulin as regulators of metabolism (17). Since IGF-II binds to the IGF-I receptor to mediate its actions, while the specific IGF-II/mannose 6-phosphate receptor is mainly involved in peptide clearance (43); most probably, the IGF-II effects in adipocytes would be similar to the ones caused by IGF-I, as observed in trout myocytes, but further experiments with IGF-II should be performed to study its role in glucose metabolism in trout adipocytes.

Fish are known to be intolerant to glucose, especially the carnivorous species. The low glucose uptake response to insulin on trout adipocytes is in agreement with this, although IGF-I could contribute to maintain glucose homeostasis in a higher degree in fish than in mammals. In addition, the adipose tissue in fish is, however, less relevant in the clearance of plasma glucose, than skeletal muscle, in front of a glucose load (2).

The relationship between metabolic effects and signal transduction pathways induced by insulin and IGF-I is still unknown in fish adipocytes. However, the use of specific inhibitors has permitted us to better understand the role of the different pathways in the process of glucose uptake. We have observed that both, PD98059 and wortmannin, inhibited the stimulation of glucose uptake by IGF-I at approximately the same level. Similar results have been found in mammalian adipocytes, in which wortmannin mainly, but also, to a lesser degree, PD98059, inhibit the stimulatory effect of insulin on glucose transport (16, 26, 44). In gilthead sea bream myocyte culture, wortmannin caused a significant decrease in glucose uptake stimulated by IGF-II, while PD98059 did not provoke such a reduction, suggesting that PI3K/AKT is the main pathway involved in glucose uptake (17).

Both pathways, MAPK and AKT, seem to be involved in the stimulation of glucose uptake by insulin and IGF-I in rainbow trout adipocytes. The finding that glucose uptake, also during basal conditions is blocked by cytochalasin B, suggests that glucose transport to the cell is mediated by specific facilitative transporters such as GLUT-1 or GLUT-4. Cytochalasin B is known to bind specifically to the facilitative glucose transporters with different affinity and has been demonstrated to inhibit glucose uptake in mammalian, but also in fish cells (19, 59). Despite this, we cannot differentiate in our study between the two glucose transporter isoforms; the existence of the insulin-responsive transporter GLUT-4 in fish adipocytes, suggests that the stimulation of the transport might be through the translocation of GLUT-4 to the plasma membrane (30, 64). In response to insulin, piscine GLUT-4 translocates to the membrane in mammalian cells and trout muscle cells in culture (9, 20). Nevertheless, further studies are needed to characterize the exact role of MAPK and AKT pathways, and the regulation of GLUT-4 translocation and glucose transport, in trout adipocytes.

**Perspectives and Significance**

Adipose tissue in fish, as in mammals, acts not only as a fat depot/mobilization tissue but also as an endocrine organ, and hence controls energy balance in the organism. Moreover, fat tissue is in continuous development, with processes that include proliferation and differentiation of new adipocytes and hypertrophy of mature cells, depending on diet and hormonal control. The adipocyte culture is an excellent tool to examine the factors and mechanisms controlling adipose tissue metabolism and adipogenesis, which are of great interest to know how fish regulate adiposity. To know how to control or manipulate the fat content in the cultured fish is one of the goals of modern aquaculture, since the usually high level of fat content in fish diets, can lead to undesirable high visceral adipose depots. Besides the inclusion of dietary vegetable ingredients can lead also to alterations in lipid metabolism, in which adipocytes play a crucial role. Nevertheless, the mechanisms underlying these processes are still not well known. Therefore, we show some data on the hormonal regulation of adipose tissue metabolism using a primary cell culture of fish adipocytes, which opens new possibilities to study the control of fish adiposity to improve dietary effectiveness and quality of cultured fish.

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DISCLOSURES
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REFERENCES


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