Expression of rainbow trout glucose transporters GLUT1 and GLUT4 during in vitro muscle cell differentiation and regulation by insulin and IGF-I

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Insulin is an anabolic hormone that plays a key role in carbohydrate metabolism, promoting the storage and synthesis of carbohydrates and inhibiting their degradation. Therefore, insulin is an essential hormone for the maintenance of glucose homeostasis (40). In mammals, skeletal muscle is the main tissue contributing to the removal of glucose from the blood to maintain normoglycemia upon insulin stimulation (50). Insulin enhances glucose uptake in skeletal muscle primarily by increasing the presence of the facilitative glucose carrier GLUT4 in the sarcolemma of muscle fibers (38). In addition to this acute effect of insulin on the subcellular localization of GLUT4, insulin stimulates the expression of GLUT4, as well as of GLUT1, in muscle and fat cells (29). GLUT4 expression is also known to increase with the progression of the myogenic differentiation process (30, 41), which entails an increase in insulin-stimulated glucose transport (30). Furthermore, the expression of GLUT4 in skeletal muscle is also stimulated by IGF-I (4), a hormone primarily involved in the control of skeletal muscle growth and differentiation (17) but also involved in metabolic regulation, evidenced by its stimulatory effects on glucose uptake in muscle cells (4).

In nonmammalian vertebrates, such as teleost fish, insulin also has an important metabolic role stimulating the entry of nutrients into the cells. Insulin is generally considered a hypoglycemic hormone in teleost fish (32), but its role regulating glucose homeostasis could be somewhat different than in mammals. In fact, the stimulatory effects of insulin on glucose uptake by skeletal muscle, which accounts for >50% of the body weight, are known only for certain species of fish (1, 42). Moreover, insulin accelerates the rates of protein synthesis and amino acid uptake to promote muscle growth (31).

Recently, insulin has been shown to directly stimulate the uptake of glucose and amino acids by trout muscle cells in culture (9) and IGF-I shares the same effects, supporting the notion that insulin and IGF-I may directly regulate carbohydrate and protein metabolism in trout skeletal muscle. In brown trout, skeletal muscle (red and white) accounts for approximately half of the total glucose uptake by tissues despite having the lowest glucose uptake rates and is the only tissue that increases its glucose uptake rate in response to a glucose load (5). Therefore, skeletal muscle can be considered a major contributor of glucose disposal from the blood in fish, and its ability to regulate its glucose uptake rate suggests the possible involvement of a regulatable facilitative glucose transport system. However, it should be noted that glucose entry into skeletal muscle could also be determined by changes in hexokinase activity and intracellular glucose concentrations (28).

Fish skeletal muscle has been shown to express several isoforms of facilitated glucose transporters. To date, four members of the family of facilitated glucose transporters (GLUT1–4) have been cloned and identified in different species of teleost fish (7, 20–22, 27, 35, 44, 45, 49). In particular, GLUT1 and GLUT4 are expressed in fish skeletal muscle (8, 12, 22, 35, 43). Functional studies of fish GLUT1 and GLUT4 homologs expressed in Xenopus oocytes have demonstrated that these transporters are indeed able to transport glucose and that they differ in their affinity for glucose, since that for GLUT4 is higher than that for GLUT1, albeit lower than that of their mammalian counterparts (7, 43). In addition, we have recently shown that insulin directly stimulates glucose uptake in trout muscle cells by increasing the abundance of GLUT4 at the plasma membrane, like in mammals (11). Nevertheless, little is known about the regulation of the expression of fish GLUT4 by insulin or IGF-I. Previous in vivo studies from our laboratory have shown a direct correlation between blood insulin levels and mRNA and protein expression levels of GLUT4 in trout red skeletal muscle (8, 12). In contrast to GLUT4, GLUT1 mRNA expression in both white and red trout skeletal muscle remained invariable to the changes in plasma insulin and IGF-I levels (39, 43).}

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insulin (8). All of these observations indicate that fish GLUT4 and GLUT1 are structural and functional homologs of mammalian GLUT4 and GLUT1, respectively.

The aim of this work was to investigate the expression of GLUT4 and GLUT1 throughout trout muscle differentiation, the regulation of the expression of these transporters by insulin and IGF-I in myoblasts and myotubes, as well as glucose uptake under basal and insulin-stimulated conditions in myoblasts and myotubes. To address this issue we used a primary culture of trout muscle satellite cells, a system that can reproduce the differentiation process taking place in skeletal muscle, shows insulin and IGF-I binding (10), and has been used to study the direct metabolic effects of insulin and IGF-I in trout muscle (9). The results from our study indicate that the basal expression of GLUT4, but not of GLUT1, markedly increases throughout the differentiation process of trout skeletal muscle cells and that the expression of both GLUT4 and GLUT1 is stimulated by insulin and IGF-I in myoblasts and myotubes.

MATERIALS AND METHODS

Animals. Rainbow trout (Oncorhynchus mykiss) of 5–10 g were purchased from the Piscifactoria Truites del Segre (Oliana, Lleida, Spain) and were maintained in the facilities of the Faculty of Biology at the University of Barcelona in a closed-water flow circuit with water at a temperature of 12°C. Animals were fed ad libitum with a commercial diet and fasted 24 h prior to the experiments. Brown trout (Salmo trutta) of 5–10 g, obtained from the Piscifactoria de Bagà (Generalitat de Catalunya; Barcelona, Spain), were maintained and fed under the same conditions as described above. The experimental protocols used for trout in this study have been reviewed and approved by the Ethics and Animal Welfare Committee of the University of Barcelona, Spain.

Isolation of trout muscle satellite cells. Animals (40 to 80 for each isolation) were killed by a blow to the head and immersed in 70% ethanol for 30 s to sterilize external surfaces. Muscle satellite cells from rainbow trout were isolated and cultured following a protocol previously described (10, 15), with some modifications. Briefly, after removal of the skin, dorsal white muscle was isolated in sterile conditions and collected in DMEM containing 9 mM NaHCO3, 20 mM HEPES, 15% horse serum, and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin B) under an air atmosphere. After 24 h of plating, plates were washed to eliminate those cells not adhered to the well. Medium was routinely renewed every 24 or 48 h. All cultures were monitored by observation with an inverted microscope (Zeiss Axiovert 25).

RNA isolation and cDNA synthesis. Total RNA from muscle cells incubated in the absence or presence of salmon insulin (kindly donated by Dr. Erika Plisetskaya) or trout IGF-I (IBT Systems, Reutlingen, Germany) was purified using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). The cDNA synthesis reaction was performed using 1 µg of total RNA and a final concentration of 5 mM MgCl2, 1 × Mg-free PCR buffer, 1 mM deoxynucleotides, 2 U/µl RNase OUT (Invitrogen, Prat de Llobregat, Spain), 2.5 U/µl murine leukemia virus reverse transcriptase (RT) (Applied Biosystems, Barcelona, Spain) and 2.5 µM random hexamers (Applied Biosystems) in a total volume of 20 µl. Tubes were incubated at room temperature for 10 min and RT was performed using a thermocycler (model PTC-200; MJ Research, Waltham, MA) at 42°C for 50 min followed by 95°C for 10 min to inactivate the RT.

GLUT4 and GLUT1 expression analysis by real-time PCR. cDNA from rainbow trout muscle cells was amplified with the LightCycler FastStart DNA Master Plus SYBR Green I kit (Roche, Sant Cugat del Vallès, Spain) by real-time quantitative PCR using a LightCycler instrument and software (Roche). cDNA was diluted 1:5 to detect GLUT4 and GLUT1, whereas it was diluted 1:400 to detect ribosomal RNA 18S. Primers used for trout GLUT4 (AF247395), GLUT1 (AF247728), and 18S (AF308735) amplification were designed using Wisconsin Package Version 9.0 (Genetics Computer Group), and their sequences are shown in Table 1. Final concentration of each primer was 0.4 µM in all cases.

The following settings in the LightCycler instrument were used to amplify GLUT4. An initial activation step of 10 min at 95°C was followed by three-step cycling (40 cycles): 5 s at 95°C (denaturation), 8 s at 60°C (annealing), and 11 s at 72°C (extension). Fluorescent data were acquired after each elongation phase for 2 s at 85°C. All steps had a temperature transition rate of 20°C/s. After amplification, a melting curve analysis from 60°C to 95°C with heating rate of 0.05°C/s with a continuous fluorescence acquisition was done. Protocols used for GLUT1 and 18S detection only differed in annealing temperature (65°C and 50°C for GLUT1 and 18S, respectively), fluorescent quantification temperature (87°C for 18S) and extension time (9 s for GLUT1 and 18S). PCR specificity was routinely checked by the melting curve obtained from the software and by agarose gel electrophoresis, where only one DNA fragment of expected size was observed in all cases. In parallel to the samples, a standard curve generated with serial dilutions of plasmid containing the target sequence (GLUT4, GLUT1, or 18S) was included in each run. Concentration values expressed as arbitrary units were assigned to each

Table 1. Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Primer Sequence</th>
<th>5’–3’ Position</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT4_Forward</td>
<td>5’-GTGCAGGCAGCTATGCTGATTATC-3’</td>
<td>151–174</td>
<td>357 pb</td>
</tr>
<tr>
<td>GLUT4_Reverse</td>
<td>5’-TAGAAGAGAGCTGCTGACAGG-3’</td>
<td>508–485</td>
<td>270 bp</td>
</tr>
<tr>
<td>GLUT1_Forward</td>
<td>5’-AATTACCGACAACGACTGCT-3’</td>
<td>307–326</td>
<td>114 bp</td>
</tr>
<tr>
<td>GLUT1_Reverse</td>
<td>5’-GAGAGGCAGCCGGAGATACCCG-3’</td>
<td>576–557</td>
<td>114 bp</td>
</tr>
<tr>
<td>18S_Forward</td>
<td>5’-CGGGCAATACAGGCTATGCTGATG-3’</td>
<td>1444–1462</td>
<td>211 bp</td>
</tr>
<tr>
<td>18S_Reverse</td>
<td>5’-GGGGCGAGGCTTATGCAAAACG-3’</td>
<td>1654–1637</td>
<td>114 bp</td>
</tr>
</tbody>
</table>
dilution cycle threshold values obtained from standards were used by the software to generate a standard curve and interpolate sample cycle threshold values. GLUT4 and GLUT1 expression in each sample was normalized using the ribosomal RNA 18S as a housekeeping gene. Real-time PCR assays were conducted in duplicate for each sample.

**Glucose uptake measurements.** Rainbow trout muscle cells, isolated and maintained as described above, were cultured for 2 and 10 days, and on the day of the experiment cells were serum starved for 4 h and subsequently preincubated in the absence or presence of human insulin (1,000 nM) for 30 min at 18°C. After the preincubation, cells were washed twice with PBS and incubated with HEPES-buffered saline containing 50 μM 2-deoxyglucose [2 μCi/ml 2-[3H]-deoxyglucose (2-[3H]-DG)] for 30 min at 18°C. After this period, transport solution was removed, and cells were rinsed three times with ice-cold PBS containing 50 mM 2-DG. Finally, cells were lysed with 0.1 N NaOH, 0.1% SDS, and the radioactivity was determined by scintillation counting using a β-counter (Packard Bioscience, Meriden, CT). Protein concentration was measured by the Bradford method (6). Nonspecific uptake was carried out with the presence of 0.45-fold over basal) (Fig. 3A). Furthermore, IGF-I significantly increased the amount of GLUT4 mRNA, although it appeared to have a maximal effect at a dose of 10 nM. GLUT1 mRNA expression also increased in response to both hormones (Fig. 3B). In the case of insulin treatment, a significant stimulatory effect on GLUT1 expression was observed only at the 1,000 nM dosage. IGF-I also caused a significant increase of GLUT1 mRNA at doses as low as 10 nM.

**Insulin and IGF-I effects on GLUT4 and GLUT1 expression in relation to the state of trout muscle cell differentiation.** The effects of insulin and IGF-I on GLUT4 and GLUT1 expression were examined at different stages during the trout muscle cell culture: at day 2 (undifferentiated cells; myoblasts) and at day 10 (differentiated cells; myotubes). Insulin (100 nM) caused a similar increase in GLUT4 expression at day 2 and at day 10 (Fig. 4A). In contrast, GLUT1 mRNA levels did not increase significantly in trout muscle cells treated with insulin after day 2 or day 10 of culture (Fig. 4B). Like insulin, IGF-I (10 nM) caused a similar increase in the expression of GLUT4 at days 2 and 10 (Fig. 4A). However, IGF-I strongly and significantly induced GLUT1 expression in trout muscle cells at day 2 (up to ~12-fold) and, to a lesser extent (~3-fold), at day 10 (Fig. 4B). It should be noted that in these experiments the basal expression of GLUT4 and GLUT1 was lower in myoblasts than in myotubes (data not shown), as observed in the cell differentiation experiments (Fig. 1).

**Glucose transport characteristics of trout muscle cells.** To examine the functional consequence of the changes in glucose transporter expression in trout muscle cells, we investigated the uptake of glucose under basal and insulin-stimulated conditions in trout muscle cells at days 2 and 10 of culture. Our results indicate that, in parallel to the increase in the basal expression of GLUT4 and, to a much lesser extent, GLUT1 in trout muscle cells, insulin-stimulated glucose uptake signifi-
significantly increased between day 2 (1.7-fold) and day 10 (2.5-fold) (Fig. 5). Basal glucose uptake did not show statistically significant differences between day 2 and day 10 (3.27 ± 0.77 and 4.62 ± 0.71 pmol 2-DG/µg protein, respectively).

DISCUSSION

In the present study, we examined the expression of the glucose transporters GLUT4 and GLUT1 throughout the course of in vitro differentiation of trout muscle cells and its regulation by insulin and IGF-I. For this purpose we used a well-characterized primary culture of trout skeletal muscle cells (10, 15, 36, 37) that provides a useful tool to reproduce the muscle differentiation process in vitro and study the biology of fish muscle cells. Importantly, trout muscle cells have receptors for insulin and IGF-I and IGF-I binding has been shown to gradually increase during myogenesis (10, 33).

The results of the present study indicate that the amount of GLUT4 mRNA gradually increased throughout the differentiation process of trout muscle cells in culture from myoblasts to myotubes, supporting our recent observation that the amount of immunoreactive endogenous GLUT4 protein in cultured trout muscle cells is higher in myotubes than in myoblasts (11). These data are in agreement with previous studies on human myosatellite cells in culture (2), which demonstrated that GLUT4 expression is higher in myotubes than in myoblasts. In the same way, Guillet-Deniau et al. (18) observed that GLUT4 mRNA was not present in muscle satellite cells isolated from rat fetuses until 11 days of culture. This feature has also been described in the mouse muscle cell line C2C12 (41). In contrast, GLUT4 expression dramatically decreased with dedifferentiation of adult rat cardiomyocytes (39). For this reason the GLUT4 gene is often used as a marker of muscle differentiation (19, 24), and the results of the present study would support the notion that the increase in the expression of GLUT4 throughout the process of muscle cell differentiation represents an evolutionary conserved feature of skeletal muscle in vertebrates.

One of the major objectives of this study was to investigate the regulation of GLUT4 mRNA levels in trout muscle cells by insulin and IGF-I. We show here that GLUT4 expression is stimulated by insulin and IGF-I in trout muscle cells and that the response is similar in cells at the myoblast and myotube stages. Our results are in agreement with previous studies using human muscle satellite cells in primary culture (3), rat cardiomyocytes (34), and fetal brown adipocytes (47). Conversely, studies using muscle and adipogenic cell lines, such as L6 or 3T3-L1 cells, respectively, have reported a downregulatory effect of insulin and IGF-I on GLUT4 gene expression (16, 26). The observed ability of insulin to stimulate GLUT4 mRNA expression in vitro in trout muscle cells is consistent with the results of a previous in vivo study from our group that established a relationship between insulin plasma levels and GLUT4 mRNA levels in skeletal muscle in trout (8). More recently, we have shown that insulin and arginine administration increase, whereas fasting decreases GLUT4 protein content in trout skeletal muscle (12), supporting the notion that circulating insulin may regulate the expression of GLUT4, both at the mRNA and protein levels, in skeletal muscle. Therefore, the present study provides the first evidence in fish (and in nonmammalian vertebrates) that insulin is able to...
regulate GLUT4 mRNA content in trout skeletal muscle by exerting its function directly on muscle cells. Furthermore, we suggest that insulin may increase GLUT4 mRNA levels in trout muscle cells by stimulating the transcriptional activity of the GLUT4 gene. Although the trout GLUT4 promoter has not yet been identified, several putative binding sites for transcription factors, such as MEF2, MyoD, and C/EBP have been found in the sequence of GLUT4 promoter from another teleost fish, Fugu rubripes (J. Morata and J. V. Planas, unpublished data). These same response elements have been identified in the mammalian GLUT4 promoter and have been involved in the tissue-specific and insulin-regulated expression of GLUT4 (14, 23, 46). Current studies in our laboratory are aimed at the identification and functional characterization of teleost GLUT4 promoter regions.

In mammals, GLUT1 is the other major GLUT isoform expressed in skeletal muscle, together with GLUT4 (25). The levels of GLUT1 mRNA increased modestly during the in vitro differentiation of trout muscle cells, in contrast to what is known in mammals (2, 18, 41, 48). In addition, the effects of insulin on GLUT1 expression did not appear to be very consistent since insulin stimulated the expression of GLUT1 in day 5 myocytes (requiring 100 nM in the time course experiments and 1,000 nM in the dose response experiments) but not in muscle cells at day 2 (myoblasts) or day 10 (myotubes). We have previously reported that GLUT1 expression in trout skeletal muscle was not affected by changes in the circulating levels of insulin (8), suggesting that insulin is not a major player in the regulation of GLUT1 expression in trout skeletal muscle. In mammalian primary cultures of adipocytes and myosatellite cells, GLUT1 expression does not change in response to an insulin treatment (3, 18, 23). Interestingly, in the trout muscle cell culture, IGF-I caused a marked increase in GLUT1 expression at day 2 and a more modest increase at day 10. Since IGF-I is also known to stimulate the proliferation of trout myoblasts (9), it is possible that the increased expression of GLUT1 could contribute to a greater fuel intake to face the increased energy demand imposed by the proliferative activity of IGF-I.

The increase in the expression of GLUT1 and, more importantly, GLUT4 during in vitro differentiation of trout muscle cells was accompanied by a significant increase in the insulin-stimulated uptake of glucose, similar to the situation in mammalian cells (30). These results suggest that the number of functional glucose transporter molecules increased in trout muscle cells during the in vitro differentiation process. Since insulin increases glucose uptake in trout muscle cells by stimulating the translocation of endogenous GLUT4 to the plasma membrane (11), the higher insulin-stimulated glucose uptake in myotubes (day 10) over myoblasts (day 2) could be explained by an increase in the amount of newly synthesized GLUT4 molecules (as suggested by the higher basal expression of GLUT4 in myotubes) that contribute to the insulin-responsive compartment and that could be mobilized to the plasma membrane in response to insulin.

**Perspectives and Significance**

The results from the present study on the differentiation-induced expression of GLUT4, and to a much lesser extent of GLUT1, and on the ability of insulin and IGF-I to directly stimulate GLUT1 and GLUT4 expression in trout muscle cells, indicate that the mechanisms regulating the expression of GLUT1 and GLUT4 in skeletal muscle are already present in fish. Due to the similarities in the regulation of GLUT1 and GLUT4 expression between fish and mammals, it is evident that these mechanisms have been remarkably well conserved.
during vertebrate evolution, underscoring the importance of the maintenance of glucose homeostasis throughout vertebrates. The present results, coupled with the known stimulation of GLUT4 translocation to the plasma membrane by insulin in trout muscle cells (11), support the idea that the relative glucose intolerance of teleost fish, and in particular of salmonid fish, may not be explained by peripheral resistance to insulin. Instead, the lower ability of fish to clear a glucose load could be due, at least in part, to the lower affinity for glucose of fish GLUT1 (43) and GLUT4 (7), compared with mammals.

GRANTS

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