Physiological regulation of the expression of a GLUT4 homolog in fish skeletal muscle

ENCARNACIÓN CAPILLA, MÓNICA DÍAZ, JOAQUIM GUTIÉRREZ, AND JOSEP V. PLANAS
Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain
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We recently cloned a glucose transporter from brown trout muscle (btGLUT) with high sequence homology to mammalian GLUT4 that is predominantly expressed in red and white skeletal muscle, the two major sites of glucose uptake in trout. To study the physiological regulation of this putative fish GLUT4, we have investigated the expression of btGLUT in red and white skeletal muscle of trout in which blood insulin levels have been altered experimentally. The expression of btGLUT in red muscle increased significantly when insulin plasma levels were elevated by either insulin or arginine treatment and decreased significantly when insulin plasma levels were reduced either by fasting or by feeding a low-protein, high-carbohydrate diet. In contrast, the expression of btGLUT in white muscle was not affected by changes in the plasma levels of insulin. These results strongly suggest that insulin may be regulating the expression of btGLUT in trout red muscle in vivo and set the ground to test the hypothesis that btGLUT may be considered a GLUT4 homolog in fish.

IN MAMMALS, THE TRANSPORT of glucose from the blood into cells is carried out by a family of glucose transporter (GLUT) proteins. The various GLUT isoforms (GLUT1–5, 8–11) share a considerable number of structural similarities but differ in their site of expression, glucose transport properties, and hormonal and metabolic regulation (8, 10, 14, 17, 22, 23).

Like mammals, fish appear to have different isoforms of glucose transporter proteins. To date, three different GLUT isoforms have been identified in fish. Our group has recently cloned a GLUT in skeletal muscle of brown trout, named btGLUT, which bears high sequence and structural homology with mammalian GLUT4, is expressed in insulin-sensitive tissues, although not exclusively, and shows the highest level of expression in skeletal muscle (24). In addition to this putative GLUT4 homolog, another transporter with homology to GLUT1, named OmmyGLUT1, has also been cloned in fish (29). More recently, a GLUT with structural similarities to mammalian GLUT2 has been identified in fish liver (13). Therefore, the identification of GLUT1, GLUT2, and GLUT4-like molecules in fish with significant structural similarities with their mammalian counterparts suggests that GLUTs are, at least structurally, evolutionarily conserved. One important question that needs to be addressed is whether fish GLUTs are also functionally similar to their mammalian counterparts. Recent studies on the heterologous expression of OmmyGLUT1 in Xenopus oocytes have shown that OmmyGLUT1 is indeed a functional facilitative GLUT with similar transport properties to mammalian GLUT1 (28). However, there is no information to date on the functional properties of the putative GLUT4 (btGLUT) and GLUT2 homologs in fish.

It is well recognized that insulin is a key factor for the maintenance of glucose homeostasis. In mammals, one of the major effects of insulin is to promote the uptake of circulating glucose into its target tissues, primarily skeletal muscle and adipose tissue. The stimulation of glucose uptake by insulin is mediated mostly by GLUT4, the insulin-sensitive GLUT, which rapidly translocates from intracellular storage sites to the plasma membrane upon stimulation by insulin (11). In addition to the acute effects of insulin on GLUT4 translocation in mammalian target tissues, insulin has been shown to stimulate the expression of GLUT4 in adipose tissue but not in skeletal muscle. In fish, insulin has also been shown to have a hypoglycemic effect at least in part by stimulating the in vivo uptake and utilization of glucose, mostly by skeletal muscle (15). Although it is not known whether insulin can stimulate glucose uptake directly in fish skeletal muscle, the presence of specific insulin receptors that can be regulated by the circulating levels of insulin (1, 2, 21) suggests that skeletal muscle may be capable of increasing its glucose uptake rate in direct response to insulin. In fact, it has been shown that administration of a glucose load in trout causes an increase in the plasma levels of insulin and a resulting increase in glucose uptake rates only in skeletal muscle (3). Therefore, the presence of a putative GLUT4 homolog in fish
skeletal muscle (24) raises the possibility that insulin could stimulate glucose uptake in this tissue by regulating the number of transporter molecules at the plasma membrane. For this reason, it will be important to determine whether the fish GLUT4 homolog (btGLUT) is regulated by insulin and, therefore, whether it is functionally similar to mammalian GLUT4.

The purpose of the present study was to investigate the in vivo effects of insulin on the expression of the putative GLUT4 (btGLUT) homolog in fish skeletal muscle and provide support for the hypothesis that btGLUT may be a functional homolog of GLUT4 in fish muscle.

MATERIALS AND METHODS

Animals

Two-year-old brown trout (Salmo trutta) from a cultured stock at the Piscifactoria de Bagà (Generalitat de Catalunya) and adult rainbow trout (Oncorhynchus mykiss) from a cultured stock at the commercial farm Truites del Segre (Lleida, Spain) were kept under natural conditions of temperature and photoperiod.

In Vivo Experiments

Fasting. Brown trout were either fed daily (control; n = 10) or deprived of food for 45 days (fasted; n = 10). In brown trout, a 45-day fast has been shown not to be life threatening and to effectively reduce plasma insulin levels (20). At the termination of the experiment, fish from the fed control and fasted groups were anesthetized in 3-aminobenzoic acid ethyl ester (0.1 g/l; Sigma, St. Louis, MO) dissolved in fresh water, immediately centrifuged, and the plasma was stored at -80°C for analysis. In fed trout, a 45-day fast has been shown not to be life threatening and to effectively reduce plasma insulin levels (20). At the termination of the experiment, fish from the fed control and fasted groups were anesthetized in 3-aminobenzoic acid ethyl ester (0.1 g/l; Sigma, St. Louis, MO) dissolved in fresh water, immediately centrifuged, and the plasma was stored at -80°C for analysis.

Blood and muscle tissue samples from the control and insulin-injected group. Twenty-four hours after the injection, whereas muscle tissue samples from both groups were collected only at 8 and 24 h after the injection, as described in Fasting.

Northern Blot Analysis

Total RNA from red and white muscle was purified with Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's specifications and resuspended in Formazol (Molecular Research Center). Total RNA samples (15 μg) were loaded onto a formaldehyde-agarose gel, transferred overnight by upward capillary elution onto a nylon membrane (Nytran, Schleicher & Schuell), and cross-linked. Membranes were prehybridized in a commercial buffer (UltraHyb, Ambion, Austin, TX) for ≥2 h in roller tubes at 42°C and subsequently hybridized overnight at 42°C with a 32P-labeled 2.3 kb cDNA fragment of btGLUT, which includes the entire coding region (nucleotides 151–2472; GenBank AF247395), as previously described (24). After membranes were stripped by incubating them twice with boiling stripping buffer (0.1× SSC-0.1% SDS) for 15 min, the blots were rehybridized with a 32P-labeled, 467-bp cDNA fragment of rainbow trout GLUT1 (OnmyGLUT1) (29), of which 354 bp correspond to the 3' untranslated region (nucleotides 1697–2163; GenBank AF247728). Finally, the blots were rehybridized with a brown trout 18S 32P-labeled cDNA fragment (24) to control for RNA loading and transfer. After each hybridization, the blots were washed three times with 2× SSC-0.1% SDS at room temperature, once with 1× SSC-0.1% SDS at 42°C and twice with 0.1× SSC-0.1% SDS at 42°C. Subsequently, the blots were exposed to Kodak X-AR film at −80°C. The intensity of the bands in films was quantified by laser scanning densitometry.

In all Northern blots with red and white muscle RNA, a single band of 3 kb was obtained with the btGLUT cDNA probe, and a single band of 2.7 kb was obtained with the OnmyGLUT1 cDNA probe. The fact that each probe detected a different mRNA species and that the OnmyGLUT1 cDNA probe hybridizes mostly with the 3' untranslated region of fish GLUT1, where there is little homology (35%) between the two fish GLUT isoforms, indicates that the probes are specific.

Assays

Endogenous plasma insulin levels were measured by a radioimmunoassay using bovine insulin as standard and radiolabeled tracer and rabbit anti-bovine insulin antiserum (9), which has been validated for trout plasma (18, 19). The levels of porcine insulin in plasma in the insulin treatment experiment were measured by a commercial radioimmunoassay kit for mammalian insulin (Schering, Madrid, Spain). Plasma glucose levels were measured by the glucose oxidase method with a commercial assay kit (Menarini Diagnostics, Firenze, Italy).

Statistical Analysis

Differences between groups were analyzed for statistical significance with the unpaired Student’s t-test or by one-way analysis of variance, followed by the Fisher’s protected least significant difference test. Results are expressed as means ± SE.
RESULTS

To test the hypothesis that insulin can regulate the expression of btGLUT in fish muscle in vivo, we examined the expression of btGLUT in white and red skeletal muscle from trout in which blood insulin levels had been manipulated experimentally.

Effects of Fasting

Fasting caused a significant ($P < 0.01$) decrease in mRNA expression of btGLUT in red muscle but not in white muscle (Fig. 1A) and was accompanied by significant ($P < 0.001$) decreases in the plasma levels of insulin and glucose (Table 1).

Effects of Diet Adaptation

Fish fed a diet with low-protein, high-carbohydrate content showed significantly ($P < 0.05$) lower expression of btGLUT mRNA (Fig. 2) in red muscle, but not in white muscle, and significantly ($P < 0.05$) lower plasma insulin levels (Table 1), despite having significantly ($P < 0.05$) higher blood glucose levels than fish fed the control diet.

Effects of Insulin Treatment

Insulin treatment increased significantly ($P < 0.05$) the amount of btGLUT mRNA in red muscle, but not in white muscle, at 24 h after the injection (Fig. 3). The plasma levels of insulin in insulin-injected fish were significantly ($P < 0.05$) higher than in control fish at 24 h after the injection; however, glucose plasma levels were not significantly different between the two groups of fish at this time point (Table 1). Although no blood samples were taken earlier than 24 h after the injection, it is likely that insulin, which is known to be

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Table 1. Effects of fasting, diet adaptation, and insulin treatment on insulin and glucose plasma levels in trout

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Insulin, ng/ml</th>
<th>Glucose, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting, 45 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.5 ± 1.8</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Fasted</td>
<td>7.1 ± 0.9†</td>
<td>4.6 ± 0.4†</td>
</tr>
<tr>
<td>Diet adaptation, 60 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>16.3 ± 1.3</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>LP-HC diet</td>
<td>12.9 ± 1.2†</td>
<td>7.7 ± 0.5†</td>
</tr>
<tr>
<td>Insulin treatment, 24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.6 ± 0.4</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>Insulin</td>
<td>7.7 ± 0.9*</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 10$ (fasting), $n = 10$ (diet adaptation), $n = 6$ (insulin treatment). LP-HC, low protein high carbohydrate. *$P < 0.05$, †$P < 0.01$ vs. control group (t-test).
hypoglycemic (15), lowered glucose plasma levels at earlier time points.

**Effects of Arginine Treatment**

Arginine treatment caused a significant \( (P < 0.05) \) increase in btGLUT mRNA expression in red muscle, but not in white muscle, 24 h after the injection (Fig. 4). However, no changes in btGLUT mRNA expression in red muscle were detected at 8 h after the injection (data not shown). The effects of arginine treatment on insulin plasma levels were fast, showing significant \( (P < 0.05) \) stimulatory effects already at 2 h after the injection, and lasted at least until 8 h after the injection, because arginine was no longer effective at 24 h (Table 2). The plasma levels of glucose were significantly \( (P < 0.05) \) lower in fish injected with arginine than in saline-injected fish only at 8 h after the injection (Table 2), coinciding with the last time point in which insulin plasma levels were elevated over those of the controls.

For comparison, we have also investigated the in vivo effects of insulin on the expression of OnmyGLUT1, the GLUT1 homolog (29), in fish skeletal muscle. The expression of OnmyGLUT1 in either red or white muscle was not affected by fasting (Fig. 1B) or by any other treatment (data not shown).

**DISCUSSION**

In the present study, we have investigated the in vivo regulation of the expression of the putative GLUT4 (btGLUT) homolog in trout skeletal muscle and have shown that the expression of btGLUT in red muscle is directly correlated with the plasma levels of insulin. In contrast, the expression of btGLUT in white muscle does not appear to be affected by changes in the plasma levels of insulin. Therefore, these results suggest that insulin could regulate the expression of btGLUT in trout red muscle in vivo. Given that changes in the steady-state levels of btGLUT mRNA expression could be determined by changes in transcription of the btGLUT gene and/or stability of btGLUT mRNA, it will be important to investigate whether insulin can stimulate the rate of transcription of btGLUT in trout red muscle in future studies. Our results on the in vivo regulation of btGLUT expression in trout red muscle by insulin plasma levels constitute the first report on the physiological regulation of the expression of a glucose transporter in fish and, to the best of our knowledge, in nonmammalian vertebrates. In birds, a GLUT4-like protein has not yet been identified or characterized at the molecular level in muscle, and therefore no information regarding the regulation of its expression is available. However, in the avian skeletal muscle, insulin has been reported to stimulate the appearance at the plasma membrane of a protein that immunoreacts with a mammalian GLUT4 antibody concomitantly with an increase in glucose transport (30).

Interestingly, the expression of btGLUT in trout white skeletal muscle was not affected by insulin plasma levels, indicating that the regulation of btGLUT mRNA expression in fish appears to be tissue specific. In mammals, the expression of GLUT4 in red and white muscle has also been shown to be differentially regulated by insulin plasma levels. However, in contrast to our findings on the expression of btGLUT in fish mus-
Table 2. Time course effects of arginine treatment on insulin and glucose plasma levels in trout

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
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</thead>
<tbody>
<tr>
<td><strong>Insulin, ng/ml</strong></td>
<td></td>
<td></td>
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<tr>
<td>Saline</td>
<td>7.7 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.9 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.4 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arginine</td>
<td>21.9 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.5 ± 1.7</td>
<td>14.8 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.9 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>Glucose, mM</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Saline</td>
<td>5.9 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.7 ± 0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.2 ± 0.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.0 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.3 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.3 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.5 ± 1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.1 ± 0.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.9 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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</table>

Values are means ± SE; n = 6. Different letters<sup>a,b,c</sup> indicate statistically significant (P < 0.05; ANOVA) differences in insulin or glucose levels among time points and treatment groups. Fish at the 0 h time point were sampled just before arginine injection.

The relatively slow time course of the effects of arginine on glucose plasma levels is consistent with the known fact that fish (particularly carnivorous trout) clear a glucose load more slowly than mammals (7, 16). For this reason, it is believed that fish are relatively glucose intolerant compared with mammals. In fish, the lower ability to clear glucose from the blood is not due to lower insulin plasma levels, because fish are known to have higher insulin levels (0.2–5 nmol/l) than mammals (15). From previous studies in our laboratory, we have suggested that the lower ability of fish to deal with a glucose load could be due partially to the lower number and activity of insulin receptors in fish skeletal muscle compared with those in mammals (21, 25). Another factor that could contribute to the relative peripheral resistance to insulin in fish is the number or activity of glucose transporter molecules in skeletal muscle cells. The results from our previous (24) and present studies indicate that fish red muscle contains at least two different GLUT isoforms, one of which (btGLUT) is structurally homologous to GLUT4, and its expression can be regulated by insulin plasma levels. Therefore, it is possible that the relative peripheral resistance to insulin in fish could be due, in addition to the lower number and activity of insulin receptors, to a lower number of GLUT molecules in the plasma membrane and GLUT1 expression levels. However, it is not clear whether the decrease in GLUT1 mRNA and protein levels in fish muscle cells is due to a decrease in GLUT1 transcription and translation in the L6 muscle cell line (12, 27). Our results suggest that insulin may not be regulating the expression of GLUT1 in trout skeletal muscle. Further studies should be conducted to determine whether insulin is able to regulate the transcriptional or translational rates of GLUT1, as well as GLUT1 protein levels, in fish muscle.

The results from the arginine treatment experiment show that the decrease in glucose plasma levels, in response to elevated insulin plasma levels, precedes the increase in the expression of btGLUT mRNA in trout red muscle. Therefore, it is possible that insulin may have acutely stimulated glucose uptake in trout muscle mostly through a nongenomic effect. The occurrence in the amino acid sequence of btGLUT of motifs that are known to be important for the internalization and the translocation of GLUT4 to the plasma membrane (24) could be suggestive of the possibility that the levels of btGLUT at the plasma membrane could have increased upon insulin stimulation and contributed to the lowering of glucose plasma levels. An important question that will need to be addressed in the future is whether the number of btGLUT molecules at the muscle cell plasma membrane can be regulated by insulin.
unpublished observations) in Xenopus oocytes has revealed that their Michaelis-Menten kinetic values are slightly higher than those for their mammalian counterparts, suggesting that fish GLUTs have indeed lower affinity for glucose than mammalian GLUTs.

In conclusion, we have shown that the expression of btGLUT in trout red skeletal muscle is altered by changes in the plasma levels of insulin. These results suggest that insulin could stimulate the expression of btGLUT in this tissue in a manner similar to the regulation of GLUT4 by insulin in adipose and cardiac tissue in mammals. Our results will set the ground to test further the hypothesis that btGLUT may not be only a structural homolog of GLUT4 but also a functional homolog as well. Furthermore, our results suggest that insulin-stimulated glucose uptake in red skeletal muscle of fish could be mediated by a GLUT4-type transporter, as has been described in mammals and birds, suggesting the existence of an important evolutionarily conserved regulatory mechanism of glucose homeostasis.

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