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# Physiological regulation of glucose transporter (GLUT4) protein content in brown trout (*Salmo trutta*) skeletal muscle

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## Summary

**In brown trout, red and white skeletal muscle express the insulin-regulatable glucose transporter 4 (btGLUT4). We have previously shown that the mRNA expression of btGLUT4 in red muscle, but not white muscle, is altered under experimental conditions designed to cause changes in the plasma levels of insulin, such as fasting, insulin and arginine administration. In order to determine whether changes of btGLUT4 expression at the mRNA level are correlated with changes at the protein level, we performed *in vivo* experiments to alter blood insulin concentrations and determined the abundance of btGLUT4 protein in trout red and white skeletal muscle by immunoblotting using an antibody to salmon GLUT4. In the present study we show that btGLUT4 protein content in red muscle decreases after fasting and increases after insulin administration. By contrast, btGLUT4 protein content in white muscle decreases after fasting but is not affected by insulin treatment. Our results show a good correlation between the changes observed in btGLUT4 protein and the previously reported changes in mRNA levels in response to alterations in circulating insulin, indicating that the regulation of btGLUT4 in brown trout takes place predominantly in the red skeletal muscle.**

Key words: GLUT4, insulin, muscle, protein, trout.

## Introduction

In contrast to mammals, teleost fish have generally been considered to be glucose intolerant (Hemre et al., 2002; Moon, 2001) in view of the fact that in various fish species a glucose load results in persistent hyperglycemia (Blasco et al., 1996; Legate et al., 2001; Palmer and Ryman, 1972; Wright et al., 1998). Since teleost fish have been shown to have functional insulin receptors and insulin involved in the postprandial regulation of circulating glucose levels (Mommsen and Plisetskaya, 1991; Planas et al., 2000c), some authors pointed to a possible lack of an insulin-regulated glucose transporter as an explanation for the glucose intolerance of teleost fish (Wright et al., 1998). However, our group has identified GLUT4-homologs in brown trout (btGLUT4) (Planas et al., 2000a) and coho salmon (okGLUT4) (Capilla et al., 2004) that are expressed in skeletal muscle and adipose tissue, among other tissues.

In mammals, GLUT4 appears to be essential for the maintenance of glucose homeostasis because it mediates the action of insulin by enhancing the uptake of glucose by peripheral tissues in postprandial conditions (Watson and Pessin, 2006). By expressing okGLUT4 in *Xenopus* oocytes, we have recently shown that okGLUT4 is a functional glucose transporter with similar biochemical properties to mammalian GLUT4 but with a lower affinity for glucose (Capilla et al., 2004). Furthermore, both btGLUT4 and okGLUT4 have been shown to be regulated by insulin, providing the first evidence for the existence of an insulin-regulated glucose transporter in fish. Our group recently reported that insulin regulates the subcellular localization of okGLUT4 when expressed in 3T3-L1 adipocytes by stimulating its translocation to the plasma membrane (Capilla et al., 2004). In addition, we have studied the physiological regulation of btGLUT4 gene expression *in vivo* in trout skeletal muscle (Capilla et al., 2002), because skeletal muscle is the most important tissue for regulated glucose uptake in fish (Blasco et al., 1996). The results from our previous studies indicate that btGLUT4 mRNA levels in the slow aerobic red muscle, but not in the fast anaerobic white muscle, correlate with the levels of insulin in the blood in brown trout. We hypothesized that insulin might regulate GLUT4 mRNA expression *in vivo* specifically in red muscle and that this may be in relation to the particular metabolic characteristics of this type of muscle in fish. However, since the physiological regulation of fish GLUT4 protein levels is not known to date in either type of muscle, it is not possible to conclude that changes in mRNA levels will result in changes in the amount of btGLUT4 protein in skeletal muscle. Therefore, the purpose of the present study was to analyze the

changes in btGLUT4 protein content in brown trout red and white skeletal muscle in different experimental situations known to alter the circulating levels of insulin and to determine whether btGLUT4 mRNA and protein levels are correlated *in vivo*.

## Materials and methods

### Animals

Two-year-old brown trout (*Salmo trutta*) from a cultured stock at the Piscifactoria de Bagà (Generalitat de Catalunya) were kept under natural conditions of temperature and photoperiod. Fish were fed a standard commercial diet for trout (Dibaq, Segovia, Spain) containing 16% carbohydrates, 42% protein and 18% lipids. The experimental protocols used for trout in this study have been reviewed and approved by the Ethics and Animal Welfare Committee of the Universitat de Barcelona, Spain.

### *In vivo* experiments

#### Fasting

Brown trout were fed daily with a commercial diet (control; *N*=6) or deprived of food for 45 days (fasted; *N*=6). This period of fasting has been shown not to be life threatening (Navarro and Gutiérrez, 1995) and effective in decreasing insulin plasma levels (Baños et al., 1999; Capilla et al., 2002; Planas et al., 2000b) and btGLUT4 mRNA expression in skeletal muscle in this species (Capilla et al., 2002). After the 45-day period, animals were anesthetized in 3-aminobenzoic acid ethyl ester (0.1 g $l^{-1}$; Sigma, Tres Cantos, Spain) dissolved in fresh water and subsequently sacrificed by a blow to the head. Tissue samples of red and white muscle were excised, collected, rapidly frozen in liquid nitrogen and stored at –80°C until processed. Red muscle samples were taken from the midsection of the lateral line and white muscle samples were taken from the anterior dorsal musculature, and care was taken to avoid cross-contaminating the two different muscle types.

#### Insulin treatment

One group of brown trout (*N*=6) received an intraperitoneal injection of porcine insulin (1.7 μg/100 g fish; Sigma) after an overnight fast, and another group (control; *N*=6) received an injection with the vehicle (saline) under the same conditions as the insulin-injected group. Although a heterologous insulin was used for this experiment, previous studies have shown that mammalian insulin can be used to study the effects of insulin in fish because it binds fish insulin receptors equally well as fish insulin (Gutiérrez et al., 1995; Gutiérrez and Plisetskaya, 1991) and because it has a similar hypoglycemic activity in fish (Plisetskaya et al., 1985). Twenty-four hours after the injection, samples of red and white muscle were obtained as described in the fasting experiment.

#### Arginine treatment

One group of brown trout (*N*=10) received an intraperitoneal injection of L-arginine (6.6 μmol $g^{-1}$ fish; Sigma) after an overnight fast. Another group of brown trout (*N*=10) received one injection of the vehicle (saline) under the same conditions as the arginine-injected group. Arginine is a potent secretagogue of insulin in salmonid fish (Baños et al., 1999; Capilla et al., 2002; Mommsen and Plisetskaya, 1991; Planas et al., 2000b). Twenty-four hours after the injection, samples of red and white muscle were obtained as described in the fasting experiment.

In all the experiments, blood samples were obtained from the caudal vein of the anesthetized fish and were immediately centrifuged at 700 ***g*** for 10 min. Plasma fractions were collected and stored at –80°C until analyzed. In the insulin treatment experiment, blood samples were obtained at 6 h and 24 h after the injection.

### Preparation of total membrane fractions from skeletal muscle

Total membrane fractions from brown trout skeletal muscle were obtained as described by Muñoz et al. (Muñoz et al., 1996). One gram of skeletal muscle was homogenized with a Polytron in 10 volumes of homogenization buffer (25 mmol $l^{-1}$ Hepes, 4 mmol $l^{-1}$ EDTA, 250 mmol $l^{-1}$ sucrose, 25 mmol $l^{-1}$ benzamidine, 0.2 mmol $l^{-1}$ phenylmethylsulphonyl fluoride, 1 μmol $l^{-1}$ leupeptin, 1 μmol $l^{-1}$ pepstatin, 1 unit $ml^{-1}$ aprotinin, pH 7.4), and the homogenate was centrifuged at 15 000 ***g*** for 20 min at 4°C. The supernatant was recovered and KCl was added to a final concentration of 0.8 mol $l^{-1}$. The supernatant was incubated for 30 min at 4°C with agitation and subsequently centrifuged at 200 000 ***g*** for 90 min at 4°C. The membrane pellet was resuspended in homogenization buffer and stored at –80°C. Protein concentration was determined by the Bradford method (Bradford, 1976).

### Electrophoresis and immunoblotting

Total membrane samples (25 μg) were diluted in Laemmli sample buffer and heated for 5 min at 95°C. Proteins were separated on 12% SDS-PAGE gels and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Madrid, Spain). After blocking in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk for 2 h, membranes were incubated with a polyclonal antibody raised against the last 15 amino acids of the carboxyl terminus of okGLUT4 (Capilla et al., 2004), diluted 1:500 in blocking buffer, for 2 h at room temperature. The secondary antibody against rabbit IgG conjugated with horseradish peroxidase (BD Biosciences, Madrid, Spain) was used at a 1:5000 dilution in blocking buffer. Immune complexes were detected using an enhanced chemiluminiscence kit (Amersham, Barcelona, Spain). The membranes were stripped and immunoblotted with a monoclonal antibody against chicken actin (Developmental Studies Hybridoma Bank, University of Iowa, USA) as a loading control. In this case the secondary antibody was anti-mouse IgM conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were quantified using TotalLab v1.11. The densitometric values of btGLUT4 protein expression were corrected to the densitometric values of the loading control (actin) for each

sample and the results were expressed as the ratio between btGLUT4 and actin.

### *Plasma glucose measurements*

Plasma glucose concentrations were determined by the glucose oxidase colorimetric method with a commercial assay kit (Menarini Diagnostics, Florence, Italy).

### *Statistical analysis*

Results are expressed as means ± s.e.m. Data were analyzed using StatView 5.0 (Cary, NC, USA). Differences between two groups were evaluated by the unpaired Student's *t*-test.

## Results

In order to investigate the physiological regulation of btGLUT4 protein levels in brown trout skeletal muscle *in vivo*, we examined the amount of btGLUT4 protein in red and white muscle in three different experimental conditions known to alter blood insulin levels and muscle btGLUT4 mRNA levels in brown trout (Baños et al., 1999; Capilla et al., 2002; Navarro and Gutiérrez, 1995; Planas et al., 2000b).

Western blot analysis performed with total membrane preparations from brown trout red and white muscle to detect btGLUT4 protein levels showed a single band of a molecular mass of approximately 50 kDa in both red and white muscle (Fig. 1A). Under normal, non-stimulated conditions, the levels of btGLUT4 protein in red muscle are approximately threefold higher than in white muscle in brown trout (Fig. 1). Therefore, btGLUT4 protein levels correlate well with the higher basal expression of btGLUT4 mRNA in red *versus* white muscle in trout reported previously by our group (Capilla et al., 2002; Planas et al., 2000a).

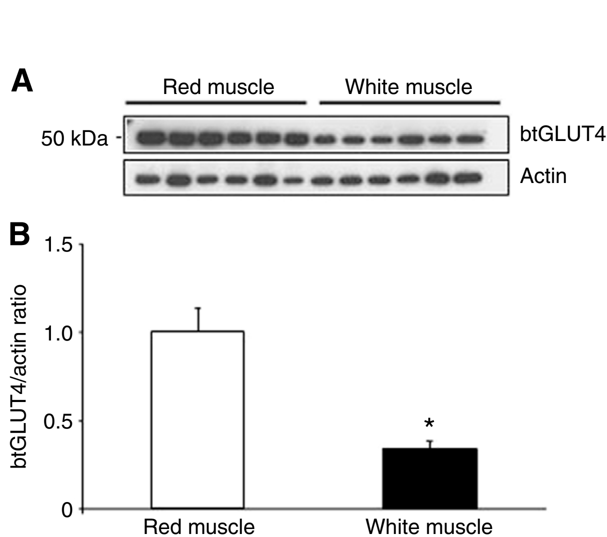


Fig. 1. Total protein content of btGLUT4 in red and white muscle from brown trout. (A) Immunodetection of btGLUT4 and actin protein in total membrane preparations from red and white muscle. 25 μg of protein was loaded in each lane, subjected to SDS-PAGE and immunoblotted using antibodies against okGLUT4 and chicken actin. (B) Densitometric analysis of the btGLUT4/actin ratios from red muscle and white muscle. Values are means ± s.e.m. and are referred to red muscle, which was set to 1 (*N*=6). *Significant differences compared with red muscle (*P*<0.01).

### *Effects of fasting*

Brown trout fasted for 45 days showed a 50% reduction in the amount of btGLUT4 protein in both red and white muscle (Fig. 2). As shown previously (Capilla et al., 2002; Navarro and Gutiérrez, 1995; Navarro et al., 1992), glucose plasma levels of fasted animals were also significantly lower than in non-fasted animals (Table 1).

### *Effects of insulin treatment*

In order to evaluate the *in vivo* effects of insulin on btGLUT4 protein levels in trout skeletal muscle, porcine insulin was administered to brown trout by intraperitoneal injection. Twenty-four hours after the injection the amount of btGLUT4 protein increased significantly in red muscle of insulin-injected trout. Conversely, btGLUT4 protein levels in white muscle were not affected by the injection of insulin (Fig. 3). The plasma levels of glucose at 6 h after the injection were significantly lower in insulin-treated fish when compared with the control fish, but no differences in plasma glucose levels were detected after 24 h (Table 1).

### *Effects of arginine treatment*

Arginine is a widely known insulinotropic amino acid in salmonids (Baños et al., 1999; Capilla et al., 2002; Planas et

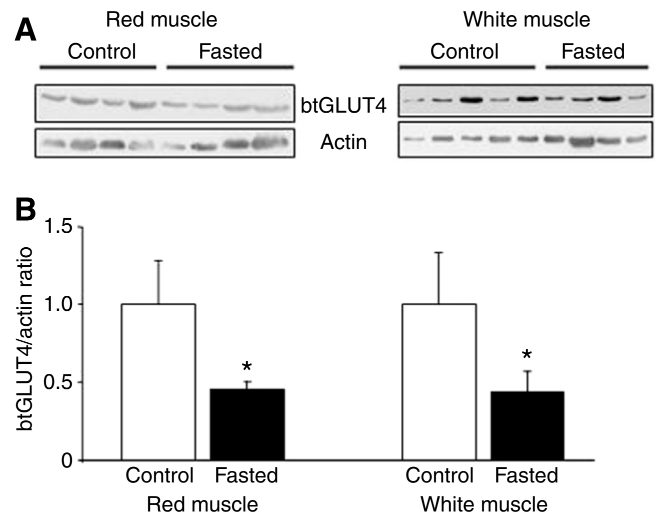


Fig. 2. Effects of fasting (45 days) on the btGLUT4 protein content of red muscle and white muscle from brown trout. (A) Immunodetection of btGLUT4 and actin protein in total membrane preparations from red and white muscle. 25 μg of protein was loaded in each lane, subjected to SDS-PAGE and immunoblotted using antibodies against okGLUT4 and chicken actin. (B) Densitometric analysis of the btGLUT4/actin ratios from red muscle and white muscle. Values are means ± s.e.m. and are referred to the fed group (control), which was set to 1 (*N*=4–5). *Significant differences compared with the control group for each type of muscle (*P*<0.01).

Table 1. *Effects of fasting, insulin and arginine treatment on glucose plasma levels in brown trout*

| Experiment | Glucose (mmol $l^{-1}$) |
|---|---|
| Fasting (45 days) | |
| Control | 4.8±0.2 |
| Fasted | 3.9±0.2* |
| Insulin treatment (6 h) | |
| Saline | 5.9±0.6 |
| Insulin | 4.1±0.3* |
| Insulin treatment (24 h) | |
| Saline | 5.1±0.8 |
| Insulin | 6.4±0.4 |
| Arginine treatment (24 h) | |
| Saline | 6.1±1.1 |
| Arginine | 9.7±1.1* |

Values are expressed as means ± s.e.m.; *N*=6 (fasting), *N*=6 (insulin treatment), *N*=10 (arginine treatment). *$P<0.05$ *versus* control or saline group (*t*-test).

al., 2000b; Plisetskaya et al., 1991). Thus, we administered arginine by intraperitoneal injection to one group of brown trout in order to determine the effects of an increase in the circulating levels of endogenous insulin on btGLUT4 protein levels in trout skeletal muscle. Arginine treatment did not significantly change the levels of btGLUT4 protein in red muscle, despite a trend towards slightly higher btGLUT4 protein levels in samples from arginine-injected fish (Fig. 4). Similarly, btGLUT4 protein levels in white muscle were not modified in arginine-injected trout. The plasma levels of glucose at 24 h after the injection were significantly higher in arginine-injected trout than in saline-injected trout (Table 1), as previously reported (Capilla et al., 2002).

## Discussion

In the present study we have analyzed the expression of btGLUT4 at the protein level in brown trout skeletal muscle *in vivo* and studied its regulation under different experimental conditions known to cause changes in the circulating levels of insulin. In brown trout red muscle, the content of btGLUT4 protein markedly decreased after fasting and increased after insulin administration. These changes in btGLUT4 protein content are similar to the previously reported changes in btGLUT4 mRNA expression in response to fasting and insulin administration in trout (Capilla et al., 2002). In comparison, GLUT4 protein levels in the mammalian red muscle also diminish after fasting (Camps et al., 1992), but either decrease or do not change in response to hyperinsulinemic conditions (Cusin et al., 1990; Postic et al., 1993).

Although plasma insulin levels were not measured in this study, it has been previously shown in brown trout, under experimental conditions similar to those used in the present study, that insulin plasma levels decrease after fasting, in parallel to glucose levels (Baños et al., 1999; Capilla et al.,

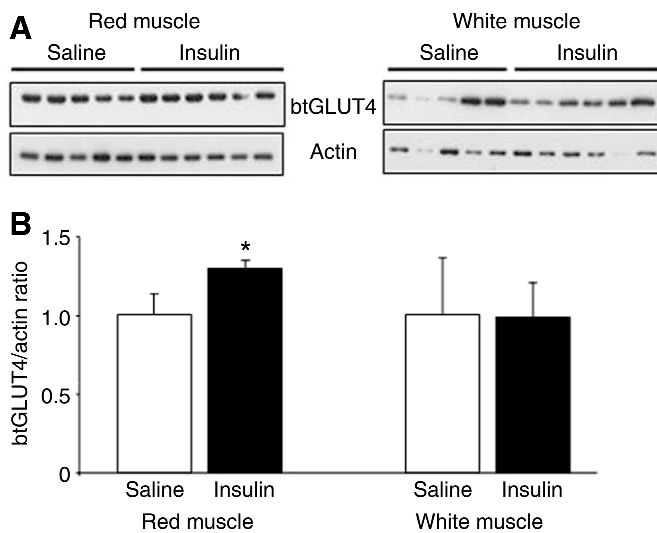


Fig. 3. Effects of insulin treatment (24 h) on the btGLUT4 protein content of red muscle and white muscle from brown trout. (A) Immunodetection of btGLUT4 and actin protein in total membrane preparations from red and white muscle. 25 μg of protein was loaded in each lane, subjected to SDS-PAGE and immunoblotted using antibodies against okGLUT4 and chicken actin. (B) Densitometric analysis of the btGLUT4/actin ratios from red muscle and white muscle. Values are means ± s.e.m. and are referred to the saline-injected group, which was set to 1 (*N*=5–6). *Significant differences compared with the saline-injected group for each type of muscle ($P<0.05$).

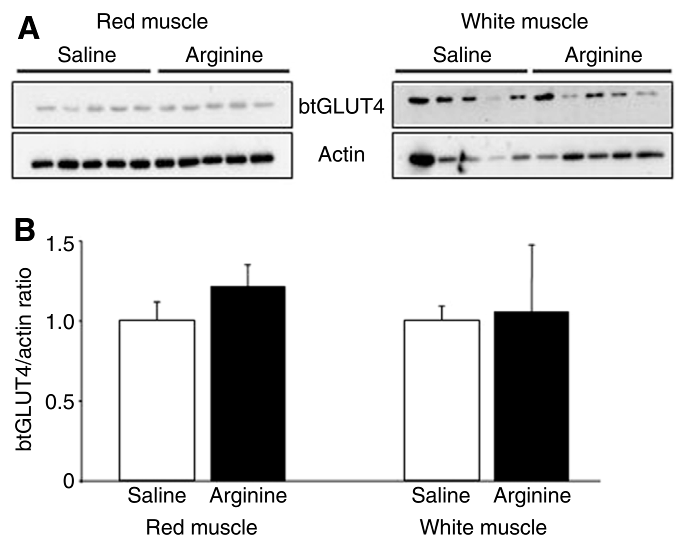


Fig. 4. Effects of arginine treatment (24 h) on the btGLUT4 protein content of red muscle and white muscle from brown trout. (A) Immunodetection of btGLUT4 and actin protein in total membrane preparations from red and white muscle. 25 μg of protein was loaded in each lane, subjected to SDS-PAGE and immunoblotted using antibodies against okGLUT4 and chicken actin. A representative western blot is shown. (B) Densitometric analysis of the btGLUT4/actin ratios from red muscle and white muscle. Values are means ± s.e.m. and are referred to the saline-injected group, which was set to 1 (*N*=10).

2002; Navarro and Gutiérrez, 1995; Planas et al., 2000b), and increase after insulin and arginine treatments, contrary to glucose levels (Baños et al., 1997; Capilla et al., 2002; Parrizas et al., 1994; Planas et al., 2000b). Therefore, the observed changes in glucose plasma levels in the present study suggest that insulin levels probably decreased after fasting and increased after insulin treatment. Our results evidence parallel changes in btGLUT4 mRNA and protein levels in brown trout red muscle which, in turn, appear to be related to changes in insulin plasma levels. By contrast, btGLUT4 mRNA and protein levels appear to change independently of glucose plasma levels. This is evidenced by the lack of a direct relationship between glucose plasma levels and the levels of btGLUT4 mRNA or protein in brown trout red muscle after insulin- and arginine-induced hypoglycemia and also after adaptation to a high-carbohydrate diet and the resulting hyperglycemia (Capilla et al., 2002). Interestingly, fasted brown trout given a glucose load show marked hyperglycemia, decreased insulin levels as well as a decreased glucose uptake rate in white and red muscle (Blasco et al., 1996), suggesting that the lowering of insulin levels by fasting may be the driving force behind the change in glucose uptake in muscle. Furthermore, post-exercise glucose utilization rates in white muscle were reported to be independent of plasma glucose levels in rainbow trout, although in red muscle this appeared to be true only at high glucose plasma levels (West et al., 1994).

Overall, these data suggest that glucose plasma levels may not be involved in the regulation of btGLUT4 expression in red muscle. Therefore, given the presence of specific insulin receptors in brown trout red muscle (Baños et al., 1997; Planas et al., 2000c) and that GLUT4 is one of the main metabolic targets of insulin in the skeletal muscle of mammals (Klip and Paquet, 1990), we believe that the observed changes in mRNA expression of btGLUT4 and in the total amount of btGLUT4 protein in brown trout red muscle could have been caused by changes in the circulating levels of insulin. In support of the hypothesis that plasma insulin could influence GLUT4 expression in brown trout red muscle, we have evidence indicating that insulin increases the level of expression of GLUT4 mRNA in primary trout muscle cells (Díaz, 2006). Further studies should be performed to determine whether insulin can directly increase the amount of btGLUT4 protein in primary trout muscle cells.

In contrast to the known stimulation of btGLUT4 mRNA expression by arginine administration in red muscle (Capilla et al., 2002), arginine did not affect the amount of btGLUT4 protein content in the present study. However, it is worth noting that arginine not only stimulates insulin secretion but also promotes glucagon release by endocrine pancreatic cells in fish (Mommsen and Plisetskaya, 1991; Navarro et al., 2002), which could explain the hyperglycemia observed 24 h after arginine administration in this and other studies (Capilla et al., 2002). Therefore, the different effects of arginine treatment on btGLUT4 protein and mRNA levels in red muscle could be due to the fact that other factors, in addition to insulin, might be involved in the regulation of btGLUT4 protein levels by affecting the rate of btGLUT4 protein degradation or the translational efficiency of btGLUT4 transcripts. Further studies should be performed to determine whether hormones such as glucagon can affect the amount of btGLUT4 protein in trout red muscle.

In this study we also examined the regulation of the content of btGLUT4 protein *in vivo* in white muscle of brown trout. In contrast to that observed in red muscle, btGLUT4 protein content in white muscle, like btGLUT4 mRNA expression (Capilla et al., 2002), was not affected by arginine or insulin treatments. However, btGLUT4 protein levels in white muscle were significantly lower in fasted trout whereas btGLUT4 mRNA content in white muscle did not change after an identical fasting period (Capilla et al., 2002), suggesting a differential regulation of btGLUT4 at the protein level in white muscle, most likely at the post-transcriptional level. In mammals, fasting also differentially affects GLUT4 mRNA and protein levels in white muscle, increasing GLUT4 mRNA levels and causing no change in GLUT4 protein levels (Camps et al., 1992), contrary to that observed in brown trout. Interestingly, the expression of cod GLUT4 has recently been shown to increase in white muscle with fasting, suggesting species-specific differences in the regulation of GLUT4 in this tissue (Hall et al., 2006).

The different *in vivo* regulation of btGLUT4 protein levels in red and white muscle of brown trout suggests that btGLUT4 is regulated in a fiber-type-dependent manner in agreement with the distinct metabolic properties of the different muscle fibers (Bone, 1978). Red muscle fibers are enriched in mitochondria and have greater oxidative capacity compared with white muscle fibers. In mammals, this correlates with a higher expression of GLUT4 protein and mRNA in red skeletal muscle, providing a greater capacity for glucose transport and insulin sensitivity (Camps et al., 1992; Kern et al., 1990; Marette et al., 1992). In the same way, brown trout red muscle also shows a higher amount of both btGLUT4 mRNA (Capilla et al., 2002) and protein (this study) compared with white muscle, along with a higher number of insulin receptors (Baños et al., 1997) and a higher glucose transport rate (Blasco et al., 1996). The results from the present study suggest the possibility that insulin levels in the blood could modulate the amount of btGLUT4 protein in red muscle and, therefore, regulate the ability of this tissue to take up glucose. Supporting this hypothesis, trout red muscle has been shown to significantly increase its glucose uptake rate after a glucose load-induced increase in circulating insulin (Blasco et al., 1996). However, white muscle also increases its glucose uptake rate after a glucose load and contributes approximately five times more than red muscle to the total glucose uptake when expressed as percent of the total body mass (Blasco et al., 1996), thus probably playing a predominant role in normoglycemia. In this context, it is interesting that in white muscle btGLUT4 protein and mRNA levels as well as GLUT1 mRNA levels do not change after insulin or arginine administration (this study) (Capilla et al., 2002). Therefore, it will be important in future studies to

elucidate which regulatory factors and transport mechanisms may underlie glucose uptake in white muscle.

In summary, this study shows that the physiological changes in the amount of btGLUT4 protein in red muscle are similar to those previously described at the mRNA level (Capilla et al., 2002) and that they may be related to blood insulin levels. Similar to what was detected at the mRNA level (Capilla et al., 2002; Planas et al., 2000a), the present study shows that the content of btGLUT4 protein is higher in red than in white muscle and that the physiological regulation of btGLUT4 takes place predominantly in red muscle.

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