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ACCIONS DE LA INSULINA SOBRE EL TRANSPORTADOR DE GLUCOSA GLUT4 EXPRESSAT EN EL MÚSCUL ESQUELÈTIC DELS PEIXOS TELEOSTIS

Memòria presentada per

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III. RESULTATS I DISCUSSIÓ

CAPÍTOL I: REGULACIÓ DEL CONTINGUT DE GLUT4 *IN VIVO* EN MÚSCUL ESQUELÈTIC DE TRUITA

REGULACIÓ DEL CONTINGUT DE GLUT4 *IN VIVO* EN MÚSCUL ESQUELÈTIC DE TRUITA

Resum

En mamífers, la insulina és una hormona clau per a mantenir la concentració de glucosa en sang dins un rang estret de valors. Durant un estat d'hiperglucèmia, la insulina és secretada per les cèl·lules endocrines del pàncrees i estimula la captació de glucosa en els seus teixits diana per tal d'eliminar l'excés de glucosa del corrent sanguini. Aquesta regulació de l'activitat transportadora de glucosa és mediada principalment pel GLUT4. En peixos la insulina també és una hormona hipoglicèmica però el mecanisme pel qual modula els nivells de glucosa circulants encara és desconegut. Hem identificat prèviament un transportador de glucosa en múscul esquelètic de truita comuna i hem demostrat que els nivells d'insulina circulants poden modular l'expressió de l'ARN missatger de GLUT4 *in vivo* en el múscul esquelètic. Tanmateix, encara no està clar si els nivells d'insulina circulants podrien també regular la quantitat de proteïna GLUT4 en el múscul esquelètic. Per tal d'abordar aquesta qüestió hem realitzat una sèrie d'experiments *in vivo* per tal d'alterar la concentració d'insulina en sang i posteriorment analitzar el contingut de GLUT4 en el múscul. Els nostres resultats indiquen que la quantitat de proteïna GLUT4 pot ser modulada pels nivells d'insulina plasmàtics en el múscul esquelètic de truita, encara que aquesta regulació és dependent del tipus de fibra muscular.

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IN VIVO REGULATION OF GLUT4 CONTENT IN TROUT SKELETAL MUSCLE

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ABSTRACT

In mammals, insulin is a key hormone for maintaining blood glucose concentration within a narrow range. In a hyperglycemic state, insulin is secreted by the endocrine pancreatic cells and enhances glucose uptake in insulin-sensitive tissues to clear the glucose excess from the vascular system. This regulation of glucose transport activity is mediated primarily by GLUT4. In fish insulin is also a hypoglycemic hormone but the mechanism(s) used to modulate glucose plasma levels is not well understood yet. We have previously identified a glucose transporter homologous to mammalian GLUT4 in skeletal muscle from brown trout and we have demonstrated that insulin plasma levels can modulate GLUT4 mRNA expression *in vivo* in skeletal muscle. However, it remains unclear if circulating insulin levels can also regulate the amount of trout GLUT4 protein in skeletal muscle. To address this issue we performed *in vivo* experiments to alter blood insulin concentration and subsequently to analyze the GLUT4 content in skeletal muscle. Our results indicate that the amount of GLUT4 protein may be modulated by insulin plasma levels in trout skeletal muscle, and that this regulation is dependent on the type of muscle fiber.

INTRODUCTION

In most animal cells, glucose entrance is mediated by a superfamily of transmembrane proteins known as glucose transporters (GLUTs). These proteins are located at the plasma membrane and allow the entrance of glucose into the cell without energy consumption. In mammals, several isoforms have been identified and characterized by their biochemical properties, tissue expression pattern and regulation (Scheepers et al., 2004; Uldry and Thorens, 2004). In particular, GLUT4 is a high-affinity glucose transporter mainly expressed in insulin-sensitive tissues such as skeletal muscle, adipose tissue and heart. GLUT4 has been shown to be essential for maintenance of glucose homeostasis since it mediates the insulin action enhancing glucose uptake by peripheral tissues in postprandial conditions (Watson and Pessin, 2006). Heterozygous disruption of GLUT4 gene in male mice leads to a severe insulin resistance and diabetes (Rossetti et al., 1997; Stenbit et al., 1997). Thus, insulin maintains normoglycemia improving glucose utilization in peripheral tissues, mainly increasing glucose transport in muscle and adipose cells. Skeletal muscle is the principal site for insulin-stimulated glucose disposal *in vivo* as it represents a high percentage of body mass (Shepherd and Kahn, 1999). Glucose uptake in this tissue is carried out primarily by GLUT4, which is the predominant glucose transporter. Insulin modulates GLUT4 action, inducing its translocation from intracellular storage sites to the plasma membrane (Rodnick et al., 1992), and also regulating its expression (McGowan et al., 1995; Zorzano et al., 2005). In some cases of insulin deficiency, as in streptozotocin-induced diabetic rats, GLUT4 expression is reduced in mammalian skeletal muscle (Bourey et al., 1990; Camps et al., 1992). In the same way, during fasting GLUT4 protein content decreases in red muscle, although it does not change in white muscle (Camps et al., 1992), demonstrating the existence of a differential regulation depending on the muscle fiber type. Therefore, GLUT4 plays an important role in glucose homeostasis and insulin resistance in mammals.

Teleost fish have been considered to be glucose intolerant and insulin resistant (Hemre et al., 2002; Moon, 2001). Several studies in different fish species have demonstrated that a glucose load results in a persistent hyperglycemia when compared to mammals (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 production involved in postprandial regulation of circulating glucose levels (Mommsen and Plisetskaya, 1991; Planas et al., 2000b), 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). Studies on okGLUT4 in *Xenopus* oocytes have demonstrated that it is a functional glucose transporter with similar biochemical properties than mammalian GLUT4 although with a lower affinity for glucose (Capilla et al., 2004). On the other hand, 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 is expressed in 3T3-L1 adipocytes (Capilla et al., 2004). Furthermore, we have studied the physiological regulation of btGLUT4 gene expression *in vivo* by insulin in trout skeletal muscle (Capilla et al., 2002), since, as in mammals, skeletal muscle is the most important tissue for glucose uptake in fish (Blasco et al., 1996). Our results demonstrated that blood insulin levels correlate with the amount of btGLUT4 mRNA in red muscle of brown trout, but not in white muscle, suggesting that insulin stimulates GLUT4 mRNA expression *in vivo*. More recent studies have demonstrated that insulin can directly stimulate GLUT4 mRNA expression in trout muscle cells *in vitro* (Díaz and Planas, unpublished observations; see chapter II). Nevertheless, the regulation of fish GLUT4 protein levels by insulin remains to be elucidated. Therefore, the purpose of the present study was to analyze the changes in btGLUT4 protein content in brown trout skeletal muscle in parallel to the changes in circulating insulin levels.

MATERIAL 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.

In vivo experiments

Fasting. Brown trout were fed daily with a commercial diet or deprived of food for 45 days. This period of fasting has been shown not to be life threatening for this species (Navarro and Gutierrez, 1995). After this period animals were anesthetized in 3-aminobenzoic acid ethyl ester (0.1 g/L; Sigma, Tres Cantos, Spain) dissolved in fresh water and subsequently killed 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^{o}C until processed. Red muscle samples were taken from the midsection of the lateral line and white muscle samples were taken from the dorsal musculature.

Insulin treatment. One group of brown trout received an intraperitoneal injection of porcine insulin (1.7 μg/100 g fish, Sigma) after an overnight fast and another group received an injection with the vehicle (saline) under the same conditions as the insulin-injected group. Previous studies have demonstrated that mammalian insulin also has a hypoglycemic action 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 received an intraperitoneal injection of L-arginine (6.6 μmol/g fish; Sigma) after an overnight fast. Another group of brown trout received one injection of the vehicle (saline) under the same conditions as the arginine-injected group. Arginine is a potent secretagogue of insulin in fish (Mommsen and Plisetskaya, 1991). Twenty-four hours after the injection, samples of red and white muscle were obtained as described in the fasting experiment.

In all the experiments, before sacrificing the animals, blood samples were obtained from the caudal vein and were immediately centrifuged at 700 x g for 10 min. Plasma fractions were collected and stored at -80^{o}C until analyzed. In the insulin treatment experiment blood samples were obtained at 6 and 24 h after the injection.

Preparation of total membrane fraction from skeletal muscle

Total membrane fractions from muscle were obtained as described by Muñoz et al. (1996). One gram of muscle was homogenized with a Polytron in 10 volumes of homogenization buffer (25 mM Hepes, 4 mM EDTA, 250 mM sucrose, 25 mM benzamidine, 0.2 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, 1 U/ml aprotinin, pH 7.4) and centrifuged at 15000 x g for 20 min at 4^{o}C. The supernatant was recovered and KCl was added to a final concentration of 0.8 M. The supernatant was incubated for 30

min at 4°C with agitation and subsequently centrifuged at 200000 x 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 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 against the C-terminal sequence of coho salmon GLUT4 (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 same membranes were also immunoreacted with a monoclonal antibody against chicken actin (Developmental Studies Hybridoma Bank, University of Iowa, United States) as a loading control. In this case the secondary antibody was anti-mouse IgM conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, California, USA). Immunoreactive bands were quantified using TotalLab v1.11. Values of btGLUT4 expression were corrected with the densitometric values of the loading control (actin) and the results were expressed as the ratio between btGLUT4 and actin.

Glucose plasma measurements

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

Insulin radioimmunoassay

Plasma insulin levels were measured by a radioimmunoassay using bonito insulin as standard and radiolabeled tracer and rabbit anti-bonito insulin antiserum (Gutierrez et al., 1984), which has been validated for trout plasma (Navarro et al., 1991).

Statistical analysis

Results are expressed as means ± SE. Data were analyzed using Statview 5.0. Differences between groups were evaluated by the unpaired Student's t-test.

RESULTS

In order to investigate the possible *in vivo* regulation of btGLUT4 protein levels by insulin in fish skeletal muscle, we examined the amount of btGLUT4 transporter in red and white muscle in three different experiments in brown trout known to cause changes in blood insulin levels (Capilla et al., 2002). It should be noted that in all experiments performed relative btGLUT4 protein content was always higher in red muscle than in white muscle (data not shown), since longer exposures were required for white muscle to obtain a detectable signal in the film.

Effects of fasting

One group of brown trout was deprived of food for 45 days while another group was fed daily. Western blot analysis carried out with total membrane preparations from red and white muscle to detect btGLUT4 showed a single band that had a molecular weight of approximately 50 kDa. Brown trout fasted for 45 days showed a 50% reduction on btGLUT4 protein in red and white muscle (Fig. 1). Glucose plasma levels of fasted animals were also diminished. A slight decrease on insulin plasma levels was observed although it was not statistically significant (Table 1).

Table 1. Effects of fasting and arginine injection on insulin and glucose plasma levels

Experiment	Insulin (ng/ml)	Glucose (mM)
Fasting, 45 days		
Control	17.2 ± 1.8	4.8 ± 0.2
Fasted	15.1 ± 1.9	3.9 ± 0.2*
Arginine treatment, 24 h		
Saline	N. D.	6.1 ± 1.1
Arginine	4.4 ± 1.1	9.7 ± 1.1*

Values are expressed as means ± SE; n=6 (fasting), n=10 (arginine treatment). *$P < 0.05$ vs. control or saline group. N.D., non-detectable

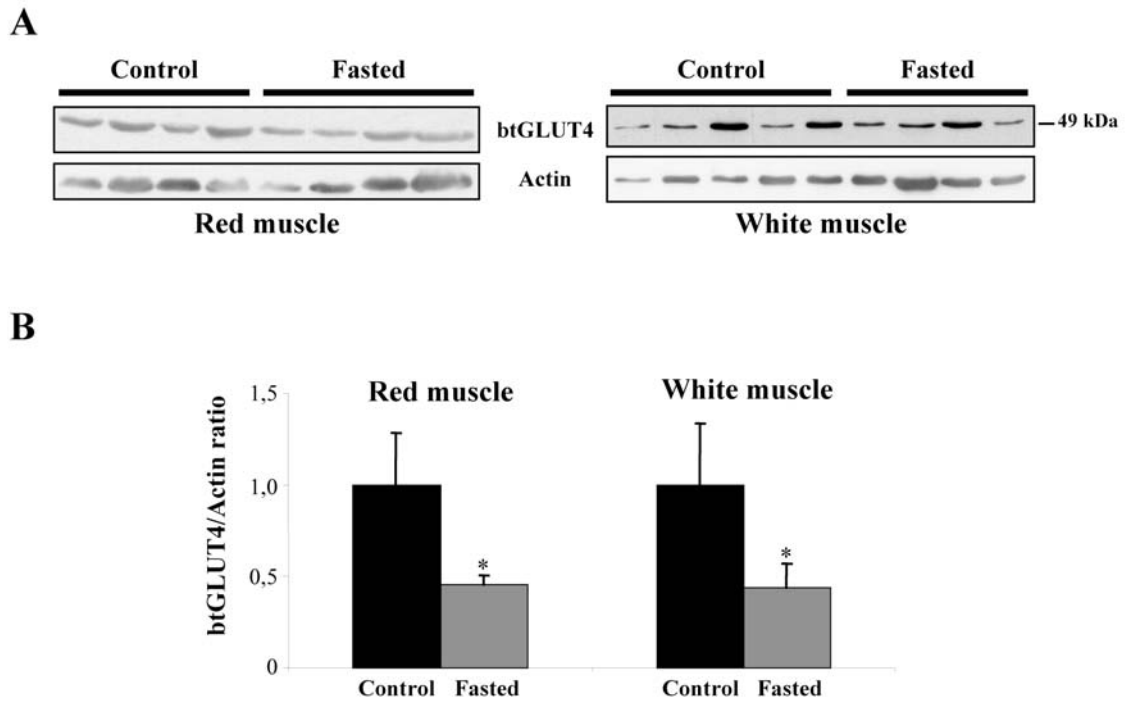

Fig. 1. Effects of fasting (45 days) on the btGLUT4 protein content of red muscle and white muscle from brown trout. *A*, Immunodetection of btGLUT4 and actin protein in total membrane preparations from red and white muscle. 25 μg of protein were loaded in each lane, subjected to SDS-PAGE and immunoblotted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

Effects of insulin treatment

In order to cause an increase in the circulating levels of insulin, an injection of porcine insulin was administered to one group of brown trout while another group received a saline injection. Twenty-four hours after the injection the amount of btGLUT4 increased significantly in red muscle of insulin-injected trout. Conversely, the btGLUT4 protein levels in white muscle did not appear to be affected by the injection of insulin (Fig. 2). Although insulin plasma levels were not determined, glucose plasma levels at 6 hours after the injection diminished in insulin-injected animals (Table 2).

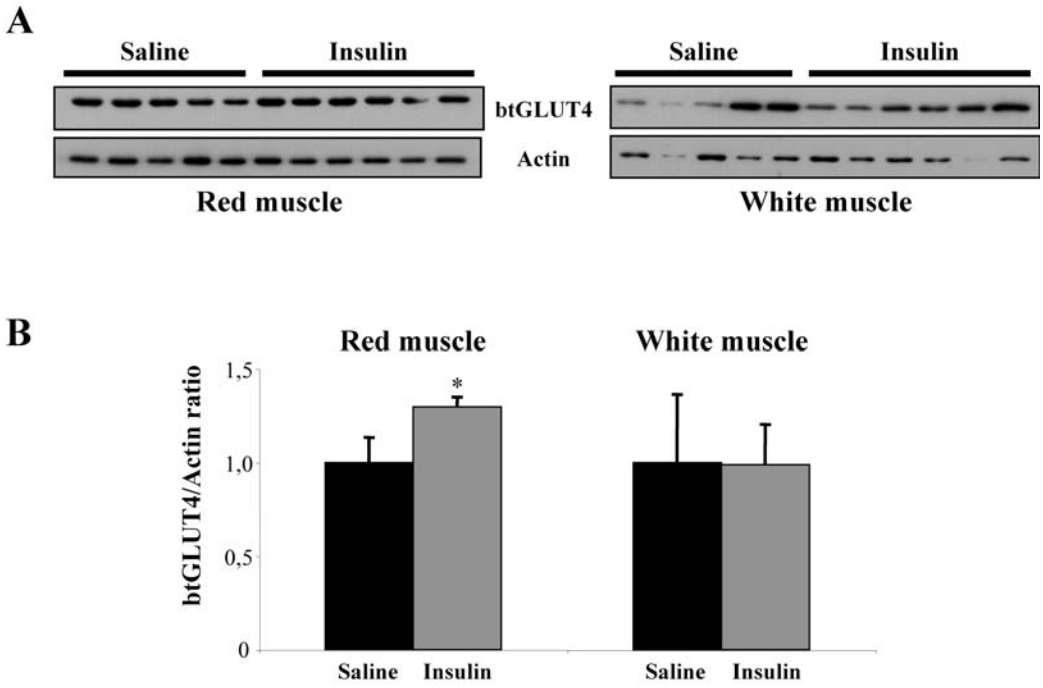

Fig. 2. Effects of insulin treatment (24 h) on the btGLUT4 protein content of red muscle and white muscle from brown trout. *A*, Immunodetection of btGLUT4 and actin protein in total membrane preparations from red and white muscle. 25 μg of protein were loaded in each lane, subjected to SDS-PAGE and immunoblotted using antibodies against okGLUT4 and chicken actin. *B*, Densitometric analysis of the btGLUT4/actin ratios from red muscle and white muscle. Values are mean ± SE and are refered to the saline-injected group which was set to 1 (n=6). *Significant differences compared to the saline-injected group ($P<0.05$).

Table 2. Effects of insulin treatment on circulating levels of glucose at 6 and 24 h after the injection

	Glucose (nM)	
	6 h	24 h
Saline	5.9 ± 0.6	5.1 ± 0.8
Insulin	4.1 ± 0.3*	6.4 ± 0.4

Values are expressed as means ± SE; n=6. *$P < 0.05$ vs. saline group.

Effects of arginine treatment

Arginine is a widely known insulinotropic amino acid in salmonids (Plisetskaya et al., 1991). Thus, we administered arginine by intraperitoneal injection to one group of brown trout in order to cause an increase in the circulating levels of endogenous insulin. Arginine treatment caused a slight increase, although not significant, in the amount of btGLUT4 molecules of red muscle (Fig. 3). In white muscle, btGLUT4 protein levels were not modified in arginine-injected trout. The plasma levels of glucose at 24 hours after the injection were significantly higher in arginine-injected trout than in saline-injected trout (Table 1). Furthermore, brown trout injected with arginine showed higher insulin plasma levels, since circulating levels of insulin in the saline-injected group were undetectable by the assay.

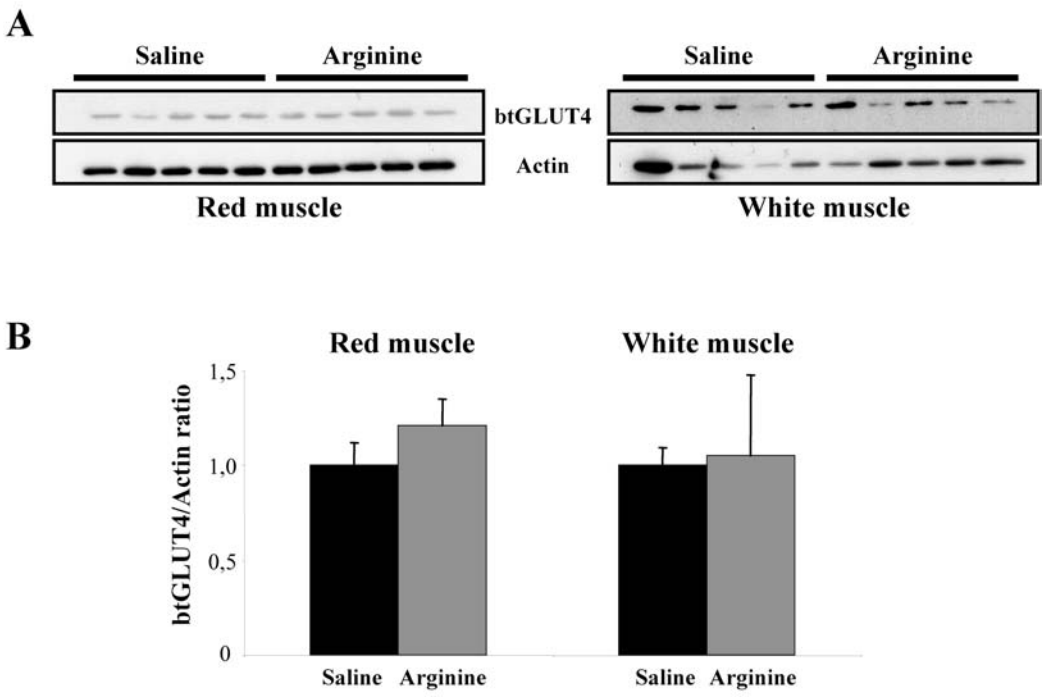

Fig. 3. Effects of arginine treatment (24 h) on the btGLUT4 protein content of red muscle and white muscle from brown trout. *A*, Immunodetection of btGLUT4 and actin protein in total membrane preparations from red and white muscle. 25 μg of protein were loaded in each lane, subjected to SDS-PAGE and immunoblotted using antibodies against okGLUT4 and chicken actin. *B*, Densitometric analysis of the btGLUT4/actin ratios from red muscle and white muscle. Values are mean ± SE and are refered to the saline-injected group which was set to 1 (n=10).

DISCUSSION

In the present study we have analyzed the expression of btGLUT4 at the protein level in trout skeletal muscle *in vivo* and its regulation by the circulating levels of insulin. Our results suggest that the circulating insulin levels may modulate btGLUT4 protein content in red muscle and, therefore, regulate the ability of glucose uptake by this tissue. Thus, after insulin treatment, the amount of btGLUT4 protein increased in trout red muscle. This increase on btGLUT4 protein agrees with that observed at the mRNA level. The amount of btGLUT4 mRNA in red muscle was also higher in insulin-injected trout (Capilla et al., 2002), indicating that insulin is likely acting at a pretranslational level. In contrast to that observed in fish, in mammals it has been reported that hyperinsulinemia *per se*, in a manner independent of glucose plasma levels, causes a reduction in GLUT4 protein in skeletal muscle, whereas it causes an increase of the transporter in adipose tissue (Cusin et al., 1990). Although insulin plasma levels were not determined in this experiment, previous reports have shown an increase in circulating insulin 24 hours after the insulin injection (Capilla et al., 2002). In addition, we have observed that insulin treatment was effective, since it had a hypoglycemic effect at 6 hours after the injection. This decrease on glucose plasma levels in insulin-injected trout is likely to be caused by an acute effect of insulin promoting the presence of GLUT4 molecules at the plasma membrane more than an effect on GLUT4 expression. Recently, we have reported that fish GLUT4 is recruited to the plasma membrane in response to insulin (Capilla et al., 2004). Furthermore, previous *in vivo* experiments have shown that the action of insulin on GLUT4 mRNA expression requires more than eight hours to be detected (Capilla et al., 2002). Therefore, insulin could have a rapid effect on the redistribution of GLUT4 to the cell surface in order to enhance glucose uptake in red muscle followed by a long-term effect on GLUT4 expression. Furthermore, we also performed an arginine treatment experiment in order to increase the circulating levels of endogenous insulin. Arginine is an important secretagogue of insulin in salmonids and, in fact, is more powerful than glucose in its ability to stimulate insulin secretion (Mommsen and Plisetskaya, 1991). In the present study, we measured plasma insulin levels only 24 hours after arginine treatment, but it is well known that insulinotropic effects of arginine are detectable from 2 h after the injection (Banos et al., 1997; Capilla et al., 2002; Parrizas et al., 1994; Plisetskaya et al., 1991). Thus, btGLUT4 protein showed a tendency towards an

increase in red muscle of animals treated with arginine, although it was not significant. This non-significant change in the amount of btGLUT4 transporter in red muscle contrasts with the rise observed in the mRNA content in response to arginine administration (Capilla et al., 2002). It should be noted that arginine not only stimulates insulin secretion, but it also promotes glucagon release by endocrine pancreatic cells (Mommsen and Plisetskaya, 1991; Navarro et al., 2002). Therefore, this effect of arginine treatment on btGLUT4 protein and mRNA levels in red muscle could be due to an additional mechanism of regulation at the translational level. Probably other factors, in addition to insulin, are involved in the regulation of btGLUT4 protein and are masking the insulin effect in red muscle, maybe affecting the rate of btGLUT4 protein degradation or the translational efficiency of btGLUT4 transcripts. On the other hand, after a period of fasting the amount of btGLUT4 markedly decreased in trout red muscle. This result agrees with previous observations at the mRNA level (Capilla et al., 2002), in which btGLUT4 mRNA content is also lower in red muscle from fasted animals. In mammals, GLUT4 protein in red skeletal muscle also diminishes after a fasting period although the mRNA levels do not change (Camps et al., 1992). Glucose plasma levels of fasted trout were lower than in fed trout as it has been previously reported (Navarro et al., 1992). It has been extensively described that a fasting period similar to that assessed in this study (45 days) causes a clear decrease on circulating insulin levels in fish (Navarro and Gutierrez, 1995). Nevertheless, in our experiment a slight decrease on insulin plasma levels was detected in fasted trout, although it was not statistically significant.

In this study we also examined the changes in the amount of btGLUT4 protein *in vivo* in white muscle of brown trout. In contrast with that observed in red muscle, btGLUT4 protein content in white skeletal muscle was not affected by the increase on plasma insulin induced by the arginine or insulin treatment. This result agrees with that observed in a previous *in vivo* study, in which btGLUT4 mRNA expression in white muscle did not change neither with arginine nor with insulin treatment (Capilla et al., 2002). However, btGLUT4 protein levels in white muscle were clearly lower in fasted trout. Conversely, btGLUT4 mRNA content in white muscle remains invariable after a fasting period (Capilla et al., 2002), indicating the existence of a differential regulation at the protein level. This result suggests that other factors could be implicated in the regulation of btGLUT4 in white muscle from fasted animals, exerting a posttranscriptional effect not detected at the mRNA level. In mammals, it has also been

reported a different effect of fasting on GLUT4 mRNA and protein in white muscle. Thus, in fasting, GLUT4 protein does not change in mammalian white muscle whereas GLUT4 mRNA is increased (Camps et al., 1992).

The different *in vivo* regulation of btGLUT4 protein levels in red and white muscle of brown trout suggests that trout GLUT4 is regulated in a fiber-type dependent manner in agreement with the distinct metabolic properties of the different muscle fibers. Red muscle fibers are enriched in mitochondria and have greater oxidative capacity compared to 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, trout red muscle also shows a higher amount of both btGLUT4 mRNA (Capilla et al., 2002) and protein (this study) compared to white muscle along with a higher number of insulin receptors (Banos et al., 1997) and a higher glucose transport rate (Blasco et al., 1996).

In summary, this study demonstrates that btGLUT4 protein content in red muscle may be modulated by blood insulin levels, although other elements could be participating in this regulation. Furthermore, it seems that changes in the amount of btGLUT4 mRNA, at least in red muscle, are correlated with similar changes at the protein level. This insulin effect promoting an increase on btGLUT4 transporters could explain the higher capacity of glucose uptake of red muscle in response to a glucose load (Blasco et al., 1996). In addition, this study provides the first evidence that insulin is exerting a differential regulation of the number of fish GLUT4 transporters depending on the muscle fiber type.

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CAPÍTOL II: ACCIÓ DE LA INSULINA SOBRE L'EXPRESSIÓ DE GLUT4 EN CÈL·LULES MUSCULARS DE TRUITA

ACCIÓ DE LA INSULINA SOBRE L'EXPRESSIÓ DE GLUT4 EN CÈL·LULES MUSCULARS DE TRUITA

Resum

La insulina és un factor important per al manteniment de l'homeòstasi de la glucosa ja que promou la captació de glucosa en els seus teixits diana durant el període postprandial. Estudis previs han demostrat que els nivells d'insulina circulants regulen l'expressió de GLUT4 *in vivo* en múscul vermell de truita. A més, es coneix que la insulina incrementa la captació de glucosa en cèl·lules musculars de peix, però el seu paper regulant els transportadors de glucosa *in vitro* encara no ha estat descrit. Així, en aquest treball hem analitzat l'expressió de GLUT4 i GLUT1 al llarg de la diferenciació de les cèl·lules musculars i la regulació de l'expressió d'aquests transportadors per insulina en un cultiu primari de cèl·lules musculars de truita. L'expressió de GLUT4 va augmentar gradualment durant el procés de diferenciació cel·lular, mentre que l'expressió de GLUT1 es va mantenir més constant durant la progressió del cultiu. La insulina va causar un increment en els nivells d'ARN missatger de GLUT4 i GLUT1, però van ser necessàries entre 12 i 18 hores d'incubació amb l'hormona per tal de poder ser detectat. A més, sembla que els efectes de la insulina sobre l'expressió de GLUT4 són dependents de l'estat de diferenciació cel·lular. Per tant, aquest treball evidencia per primera vegada que la insulina actua directament sobre les cèl·lules musculars de truita per a regular el nombre de transportadors de glucosa i així promoure l'activitat transportadora de glucosa en aquestes cèl·lules.

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INSULIN ACTION ON GLUT4 EXPRESSION IN TROUT MUSCLE CELLS IN CULTURE

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ABSTRACT

Insulin has been shown an important factor for the maintenance of glucose homeostasis enhancing glucose uptake in its target tissues during the postprandial period. Previous studies demonstrated that circulating levels of insulin regulate GLUT4 expression *in vivo* in red skeletal muscle from trout. Furthermore, it is known that insulin increases glucose uptake in fish muscle cells but its role regulating glucose transporters *in vitro* has not been understood yet. Thus we investigated the expression of GLUT4 and GLUT1 throughout muscle cell differentiation and their regulation by insulin in a primary culture of trout muscle cells. GLUT4 expression gradually increased during the differentiation process, whereas GLUT1 expression was more invariable during culture progression. Insulin provoked an increase on mRNA levels of both GLUT4 and GLUT1, but it was required between 12 and 18 hours of incubation with the hormone to be detected. Furthermore, it seems that insulin effects on GLUT4 expression are dependent on the differentiation state. Therefore, this work provides the first evidence that insulin acts directly on trout muscle cells to regulate the amount of glucose transporters to enhance glucose transport activity in these cells.

INTRODUCTION

Insulin is an anabolic hormone that plays a key role in metabolism, promoting the storage and synthesis of carbohydrates, lipids and proteins and inhibiting their degradation. In addition insulin is the most important hormone controlling glucose homeostasis. After a meal, this hormone is secreted by the pancreas and stimulates the peripheral tissues, mainly muscle and adipose tissue, to take up glucose from the blood (Shepherd and Kahn, 1999). In mammals, skeletal muscle is the major tissue that contributes to absorb the glucose from the vascular system to maintain the normoglycemia upon insulin stimulation (Zorzano et al., 2005). Insulin enhances glucose uptake in mammalian skeletal muscle mainly increasing the presence of facilitative glucose carriers in the sarcolemma of muscle fibers (Rodnick et al., 1992). These glucose transporters, known as GLUTs, are expressed in all cells and mediate the glucose entrance into the cell. In particular, GLUT4 is the most abundant isoform in adult skeletal muscle of mammals and is responsible for mediating the insulin-stimulated glucose uptake (Pereira and Lancha, 2004; Zorzano et al., 2005). Thus, GLUT4 is located in intracellular stores of insulin-sensitive tissues in the basal state and in response to insulin translocates to the plasma membrane where exerts its function (Kahn, 1996). In addition to this acute effect of insulin on the subcellular localization of GLUT4, insulin also regulates GLUT4 expression in muscle and fat cells of mammals (McGowan et al., 1995). Several studies performed on *in vitro* systems have examined the effects of insulin in GLUT4 expression with conflicting results. While insulin has been shown to stimulate GLUT4 expression in primary cultures of adipocytes and muscle cells (Al-Khalili et al., 2005; Valverde et al., 1999), two studies have reported a decrease on GLUT4 mRNA levels after insulin treatment in muscle or adipogenic cell lines (Flores-Riveros et al., 1993; Koivisto et al., 1991), . GLUT1 is the other major isoform expressed in mammalian skeletal muscle, although is more predominant during fetal life and its expression is markedly repressed perinatally (Santalucia et al., 1992). GLUT1 expression is also modulated by insulin in muscle and adipose tissue. Several studies demonstrate that insulin is able to increase the amount of GLUT1 mRNA in adipogenic and muscle cell lines by increasing the GLUT1 gene transcription rate (Garcia de Herreros and Birnbaum, 1989; Walker et al., 1989), although it has also been

shown that insulin does not affect the GLUT1 mRNA content in primary cultures of adipocytes and muscle cells (Guillet-Deniau et al., 1994; Hernandez et al., 2003).

Similarly, in fish, insulin has an important role in metabolism stimulating the entrance of nutrients into the cells. Insulin acts as a hypoglycemic hormone in fishes, promoting glucose uptake by its target tissues, primarily skeletal muscle. Moreover, insulin accelerates the rates of protein synthesis and amino acid uptake in order to promote muscle growth (Mommsen, 2001). Recently, the direct metabolic effects of insulin have been shown by its stimulation of glucose and amino acid uptake in trout muscle cells in culture (Castillo et al., 2004). Trout skeletal muscle, as the main important tissue contributing to the glucose disposal from blood (Blasco et al., 1996) has been shown to express several isoforms of facilitated glucose transporters. So far, four members of GLUT family (GLUT1-4) have been cloned in different species of teleost fishes (Capilla et al., 2004; Hall et al., 2004; Hall et al., 2005; Krasnov et al., 2001; Planas et al., 2000a; Teerijoki et al., 2000; Teerijoki et al., 2001b; Zhang et al., 2003) and, in particular, GLUT1 and GLUT4 have been shown to be expressed in trout skeletal muscle (Capilla et al., 2002). Functional studies of GLUT1 (OnmyGLUT1) and GLUT4 (okGLUT4) homologs expressed in *Xenopus* oocytes have demonstrated that these transporters differ in their affinity for glucose, being that for GLUT4 higher than that for GLUT1, but that their affinity for glucose is lower than their mammalian counterparts (Capilla et al., 2004; Teerijoki et al., 2001a). In addition, okGLUT4 is able to translocate to the plasma membrane in response to insulin when expressed in 3T3-L1 adipocytes (Capilla et al., 2004). Nevertheless, little is known about the regulation of the expression of these transporters by insulin. A previous *in vivo* study from our group recently demonstrated that blood insulin levels regulate GLUT4 expression in red muscle, but not in white muscle, of brown trout (Capilla et al., 2002). In contrast to GLUT4, GLUT1 mRNA expression remained invariable to the changes in plasma insulin both in white and red muscle. All 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 GLUT4 and GLUT1 expression throughout trout muscle differentiation as well as the regulation of gene expression of these transporters by insulin and IGF-I. To address this issue we used a primary culture of trout muscle satellite cells. This type of approach offers the possibilities of assess the direct action of the hormones into the cells without the interference existing in the *in*

vivo systems. Likewise this system reproduces all the features that occur during the muscle differentiation process. Therefore this primary culture is suitable to carry out this kind of study. Recently, Castillo et al. (2004) described the important role of insulin and IGF-I on the metabolism of trout muscle cells. Thus, we were interested to know whether or not these hormones also have an effect on the expression of glucose transporters.

MATERIAL AND METHODS

Animals

Rainbow trout of 5-10 g were obtained from the Piscifactoria Truites del Segre (Oliana, Lleida) 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^{o}C. Animals were fed *ad libitum* with a commercial diet and fasted 24 h prior to the experiments.

Isolation of muscle satellite cells from rainbow trout (*Oncorhynchus mykiss*)

Animals (40 to 80 for each culture) were sacrificed by a blow to the head and immersed in 70% ethanol for 30 seconds in order to sterilize external surfaces. Muscle satellite cells were isolated and cultured following a protocol described previously (Castillo et al., 2002; Fauconneau and Paboeuf, 2000) with slight modifications. After removal of the skin, dorsal white muscle was isolated in sterile conditions and collected in DMEM medium containing 9 mM $NaHCO_3$, 20 mM Hepes, 15% horse serum and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B, 75 μg/ml gentamycin). After mechanical dissociation of the muscle in small pieces, the tissue was enzymatically digested with a 0.2% collagenase solution in DMEM for 1 h at 18^{o}C with gentle shaking. The suspension was centrifuged (300 x g for 10 min at 15^{o}C) and the resulting pellet was subjected to two rounds of enzymatic digestion with a 0.1% trypsin solution in DMEM for 20 min at 18^{o}C and gentle agitation. After each round of trypsinization the suspension was centrifuged and the supernatant was diluted in 4 volumes of cold DMEM supplemented with 15% horse serum and the same antibiotic-antimycotic cocktail mentioned before. After two washes

with DMEM, the cellular suspension was filtered through 100 and 40 μm nylon filters. Cells were counted and cultured on 6-well plates (NUNC, Roskilde, Denmark) at a density of 3-4x10^6 cells/well. Plates were previously treated with poly-L-lysine and laminin to facilitate satellite cell adhesion. Media and chemicals used in the isolation procedure were obtained from Sigma (Tres Cantos, Spain).

Cell culture

Cells were maintained at 18^{o}C with DMEM containing 9 mM $NaHCO_3$, 20 mM Hepes, 10% fetal bovine serum and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B). After 24 h of plating, plates were washed to eliminate those cells not adhered to the well. Medium was routinely renewed each 24 or 48 h.

All cultures were morphologically monitored by observation with an inverted microscope in order to control the state of the cells.

Total RNA isolation

Total RNA from muscle cells was purified using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). This method is based on the cell lysis with a solution that contains a high concentration of caotropic ions responsible for the inactivation of RNAses. Furthermore, this solution confers conditions that favour RNA adsorption to a silica membrane. DNA was eliminated by a DNAse solution that it is added directly to the membrane during the purification. Following several washes to discard salts and other metabolites, RNA was eluted with 30 μl of RNAse-free water.

cDNA synthesis

The cDNA synthesis reaction was performed using 1 μg of total RNA and a final concentration of 5 mM $MgCl_2$, 1x Mg-free PCR buffer, 1 mM dNTPs, 2 U/μl RNase OUT (Invitrogen, Prat de Llobregat, Spain), 2.5 U/μl MuLV 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 reverse transcription was performed using a thermocycler (PTC-200, MJ Research, Waltham, United States) at 42^{o}C for 50 min followed by 95^{o}C for 10 min to inactivate the RT.

GLUT4 and GLUT1 expression analysis by real-time PCR

a) Theory of real-time PCR

The real-time polymerase chain reaction (PCR) is based on the quantification of double-stranded DNA that it is synthesized at each cycle by its labeling with a fluorescent dye. Thus, during PCR progression the increment in fluorescence due to double-stranded DNA accumulation can be observed. Several strategies exist to label nascent DNA with fluorescence. In this study we used the SYBR Green method. SYBR Green is a compound that emits fluorescence when it binds to double-stranded DNA (Fig. 1). This binding is independent of size or sequence, and for this reason the PCR reaction has to be strictly specific to amplify only one DNA fragment. Because the PCR product accumulates along the cycles, more dye it binds and more fluorescence is emitted; therefore, fluorescence intensity will be proportional to double-stranded DNA concentration.

PCR can be divided in different phases. The first phase includes the initial cycles at which PCR is just beginning and emission of fluorescence at each cycle has not raised above background. Next there is an early exponential phase in which the amount of fluorescence has exceeded a threshold fluorescence that is significantly higher than background. The cycle where this occurs is called Ct ("threshold cycle") or Cp ("crossing point"), and depends on the amount of DNA template present at the beginning of PCR. Thus, the more template DNA is present in the reaction, the faster the reaction will reach this point and the lower the Ct or Cp values will be. The Ct or Cp values are commonly used to express the experimental results. Subsequently, there is a log-linear phase in which amplification is optimal and the amount of DNA doubles at each cycle. Finally there is a plateau phase, when reaction substrates are limited and DNA amplification is more difficult.

Thus, real-time PCR technique represents a quantitative approach and is also more sensitive than conventional PCR.

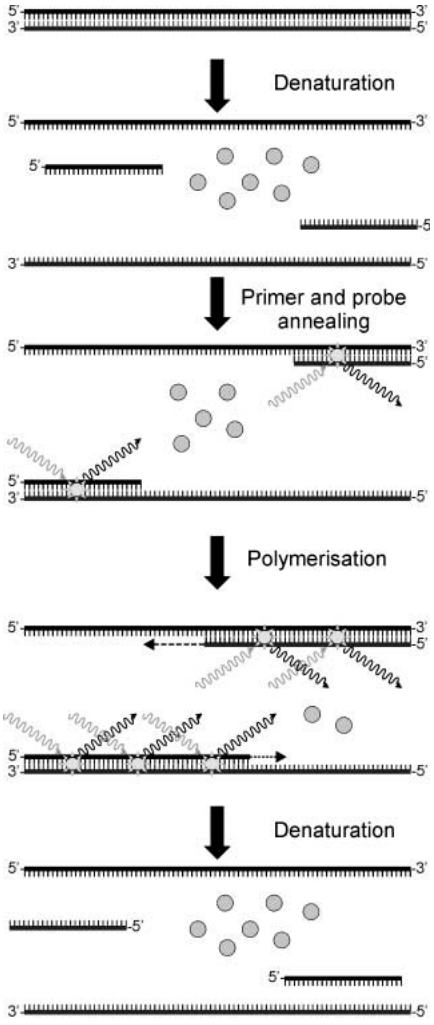

Fig. 1. Diagram of real-time PCR using SYBR Green method. During primer annealing some SYBR Green molecules (gray circles) bind to double-stranded DNA and are able to emit fluorescence, but it is in the polymerization step when more dye is incorporated to the nascent DNA and the increase on fluorescence can be monitored in real-time (Bustin, 2000).

b) Real-time PCR reaction

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) in a 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 ribosomic 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.

Table 1. - Primers used for real-time PCR

Gene	Forward	5'-3'position	Amplicon size
GLUT4_Forward	5' GTGCCAGGCTTATTGTCCATATTC 3'	151-174	357 pb
GLUT4_Reverse	5' TAGAGAAGATGGCTACCGACAG 3'	508-485	
GLUT1_Forward	5' AATATCGACAAGCCACGCTG 3'	307-326	270 pb
GLUT1_Reverse	5' GAGAAGGAGCCGAAGATACC 3'	576-557	
18S_Forward	5' CGAGCAATAACAGGTCTGTG 3'	1444-1462	211 pb
18S_Reverse	5' GGGCAGGGACTTAATCAA 3'	1654-1637	

Table 2 shows the protocol used for GLUT4 amplification. Protocols used for GLUT1 and 18S detection only differed in annealing temperature of amplification period (65^{o}C and 50^{o}C for GLUT1 and 18S, respectively), fluorescence quantification temperature (87ºC 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 dilution. Ct values obtained from standards were used by the software to generate a standard curve and interpolate sample Ct values. To normalize GLUT4 and GLUT1 expression in each sample, ribosomic RNA 18S detection was used as a control. Finally, the mRNA expression levels of target genes were expressed in relation to those of 18S. Real-time PCR assays were conducted in duplicate for each sample, and a mean value was used to calculate mRNA levels.

Statistical analysis

Differences in expression levels of GLUT4 and GLUT1 mRNA between various subgroups were evaluated by Student's t-test using SPSS software.

Table 2. - PCR protocol introduced in LightCycler instrument

	Cycles	Segment	Temperature	Time	Acquisition mode
Pre-incubation	1				
			95°C	10 min	
Amplification	40				
		Denaturation	95°C	5 s	
		Annealing	60°C	8 s	
		Extension	72°C	11 s	
		Quantification[1]	85°C	2 s	Single
Melting curve	1				
		Denaturation	95°C	10 s	
		Annealing	60°C	30 s	
		Melting[1, 2]	95°C	0 s	Continuous
Cooling	1				
			40°C	30 s	

[1] Points in which fluorescence is measured.

[2] Temperature transition rate between annealing and melting segments was 0.05°C/s, which allowed measuring fluorescence in a continuous manner. In the other steps temperature transition rate was 20°C/s.

RESULTS

Determination of GLUT4 and GLUT1 relative expression using real-time PCR

A rapid and highly sensitive real time PCR assay was developed to detect changes in GLUT4 and GLUT1 expression in trout muscle cells under different experimental conditions. Real-time PCR technique allows quantification of gene expression more accurately than other methods such as northern blot or RNAse protection assay. Serial dilutions of plasmids containing target sequences for GLUT4, GLUT1 and 18S were prepared and real-time PCR for each gene was performed. For all genes, the primer pairs used amplified only one cDNA fragment of the expected size, as can be observed by agarose gel electrophoresis and melting curve (Fig. 2). Higher initial concentrations of cDNA amplified earlier and, therefore, had a lower Ct value. The Ct values were plotted against the initial concentration to produce the standard curve and

mRNA or rRNA concentration in all the samples was determined within the linear range.

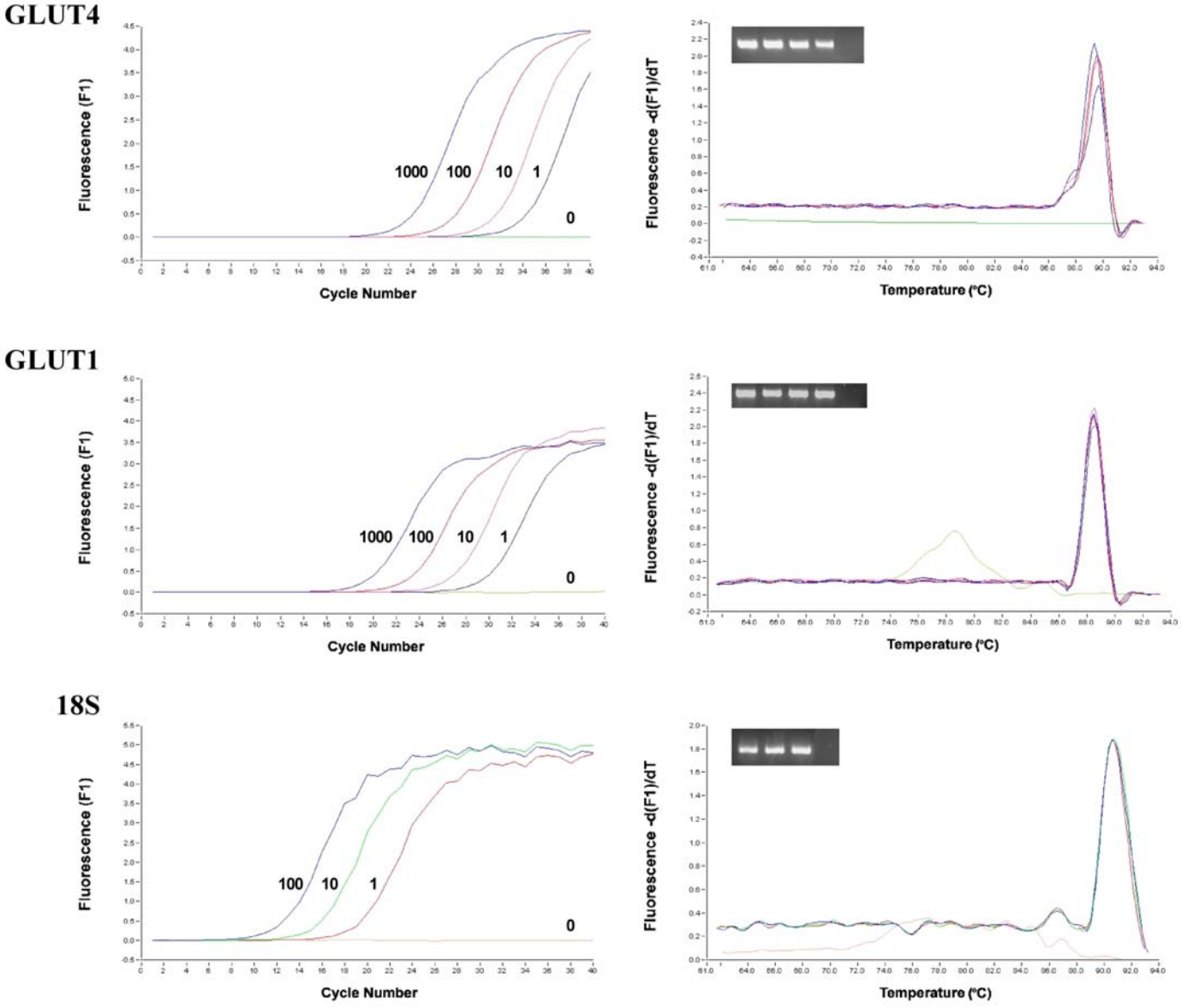

Fig. 2. Amplification plots (left panels) and melting plots (right panels) of the GLUT4, GLUT1 and 18S from real-time PCR. Each amplification plot shows different curves derived from a different amount (expressed as relative units) of standard added in the reaction. Right panels show the corresponding melting curves in which there is only one peak corresponding to the amplified DNA fragment. *Inset*, Agarose gel electrophoresis of the corresponding reactions.

GLUT4 and GLUT1 expression during trout muscle cell differentiation

During the primary culture of muscle cells from rainbow trout a morphological change in shape can be observed due to the differentiation process that these cells undergo (Fig. 3). During the first days of culture (Fig. 3A, B), cells are mononucleated with spindle-shape morphology and are called myoblasts. On day 4 (Fig. 3B) cells are more elongated and start to fuse to form small multinucleate cells called myotubes. Subsequently, these small myotubes fuse to produce large myotubes that can be observed after 8 or 10 days of culture (Fig. 3D, E). In mammals this differentiation process also involves an expression activation program of muscle-specific genes such as those implicated in muscle contraction, carbohydrate metabolism or amino acid transport (Moran et al., 2002). During the differentiation of fish muscle cells, the induction of myogenic genes such as myogenin (Rescan et al., 1995) or myosin heavy chain (Gauvry and Fauconneau, 1996) has been described.

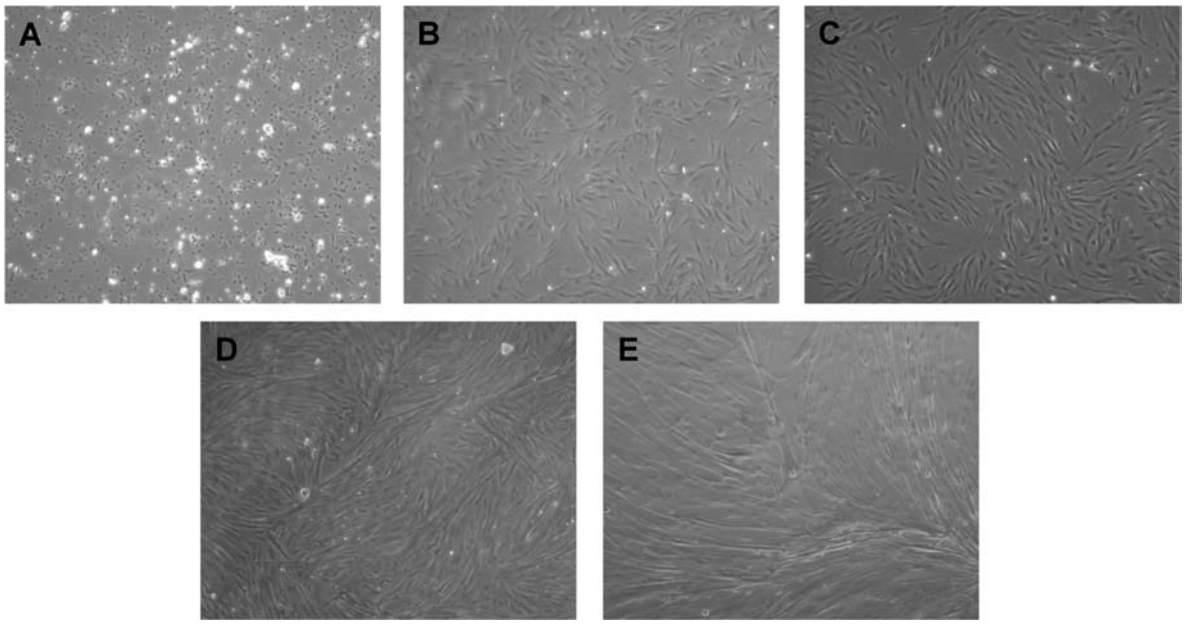

Fig. 3. Differentiation of muscle satellite cells from rainbow trout cultured at 18°C with DMEM containing 10% FBS. *A*, day 1. *B*, day 4. *C*, day 6. *D*, day 8. *E*, day 10.

Using the real-time PCR assay changes in GLUT4 and GLUT1 mRNA levels were examined throughout trout myoblast differentiation. In all cultures, an increase of GLUT4 expression was observed in parallel with the progression of cell differentiation. GLUT4 expression was already significantly increased at day 4 or 6 of culture and reached maximal expression at day 8 or 10 (Fig. 4). The magnitude of this change on GLUT4 expression between non-differentiated (day 1) and differentiated cell (day 10) differed among the different cultures, ranging between 8 and 56-fold, respectively.

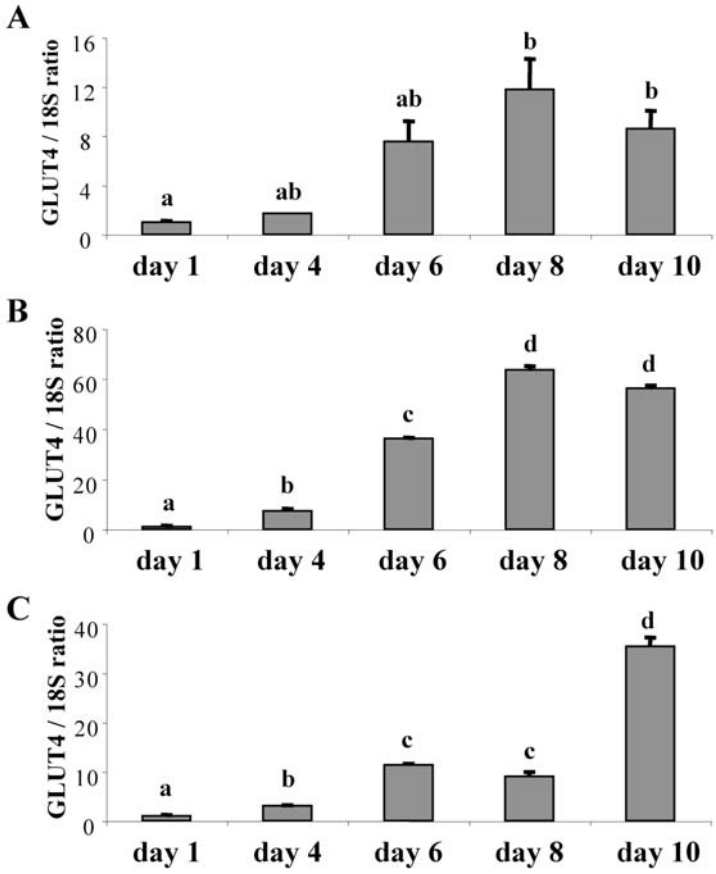

Fig. 4. GLUT4 expression at different days of culture. Each graph (A-C) corresponds to an independent experiment. Results are mean ± SE of each sample analyzed for duplicate, which were set to 1 at day 1. Different letters indicate significant differences (P<0.05).

In contrast, the expression of GLUT1 was more constant than the expression of GLUT4 throughout the trout muscle cell differentiation period (Fig. 5). In two out of three experiments, a significant increase in the amount of GLUT1 mRNA was observed after day 4. The observed increase in GLUT1 mRNA during muscle cell differentiation, which reached a maximal value at 2.4-fold over day 1, was much more modest than that of GLUT4.

In general, the relative GLUT1 expression appeared to be higher than GLUT4 expression, but the ratio between GLUT1 and GLUT4 expression decreased considerably at the final stage of myocyte differentiation (data not shown).

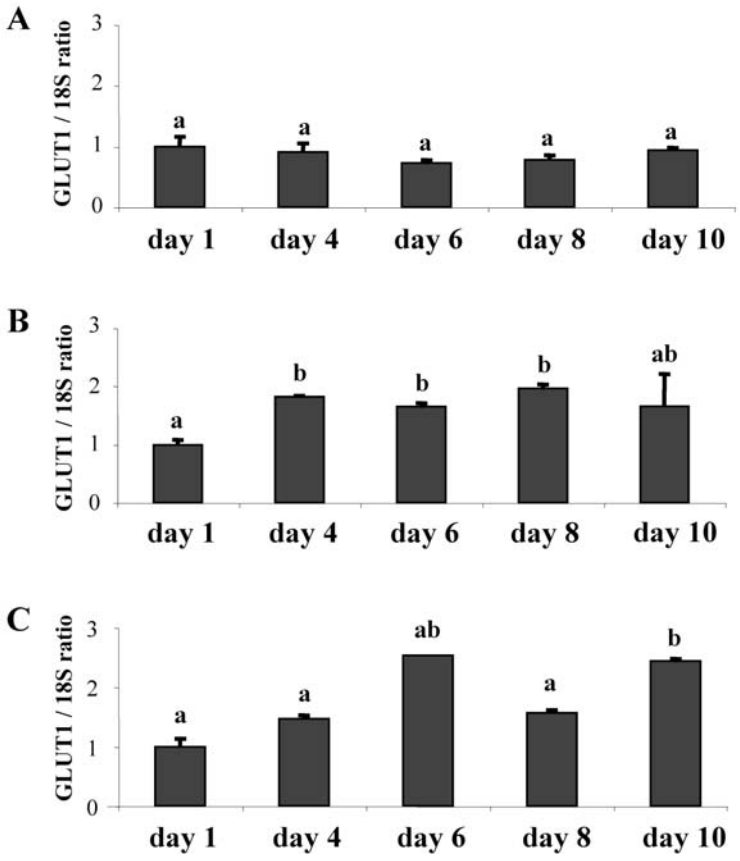

Fig. 5. GLUT1 expression at different days of culture. Each graph (A-C) corresponds to an independent experiment. Results are mean ± SE of each sample analyzed for duplicate, which were set to 1 at day 1. Different letters indicate significant differences (P<0.05).

Time course of insulin treatment on GLUT4 and GLUT1 expression in trout muscle cells

In order to study how insulin affects GLUT4 and GLUT1 expression over time, trout muscle cells at day 5 of culture were serum-deprived for 4 hours and incubated in the absence or presence of salmon insulin (100 nM) during different periods of time (3, 6, 12 and 18 hours).

An increase of GLUT4 mRNA levels in cells treated with insulin was already detectable at 6 hours, although it was not statistically significant until 18 hours of incubation (Fig. 6A). After 12 hours of insulin incubation a significant increase on GLUT1 expression was achieved and this increase was higher after 18 hours (Fig. 6B). Insulin elicited a similar increase in the expression of GLUT4 and GLUT1 at 18 hours of incubation (2.6-fold).

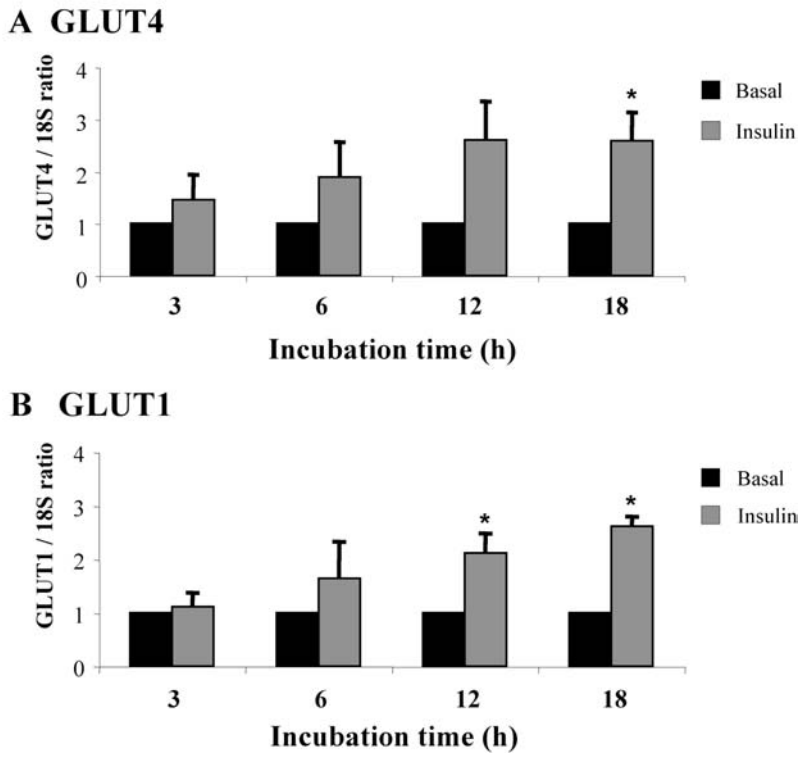

Fig. 6. GLUT4 and GLUT1 expression in response to different times of exposure to insulin. After 5 days of culture muscle cells were serum-starved for 4 h following an incubation in the absence or presence of 100 nM salmon insulin for a different period of time. At the end of the incubation time, cells were collected and processed as described in the Materials and methods section. Results are mean ± SE compared to the basal state, which was set to 1. *A*, GLUT4, Results from three independent experiments (except at 3 h which were two). *B*, GLUT1, Results from three independent experiments. * Significant differences compared to basal of each time (P<0.05).

Dose response of insulin and IGF-I on GLUT4 and GLUT1 expression in trout muscle cells

After 5 days of culture, cells previously deprived of serum were incubated with increasing concentrations of insulin and IGF-I during 18 hours and GLUT4 and GLUT1 relative expression was quantified. GLUT4 expression increased in cells treated with insulin in a dose dependent manner and achieved its maximum levels when cells were incubated with 1000 nM insulin (4.83 ± 0.45 fold over basal) (Fig. 7). IGF-I also increased the amount of GLUT4 mRNA, but its effects were not significant at concentrations below 100 nM. Although it appeared that IGF-I could be more potent than insulin stimulating GLUT4 expression at intermediate doses (10 nM and 100 nM), no significant differences between the effects of both peptides were detected.

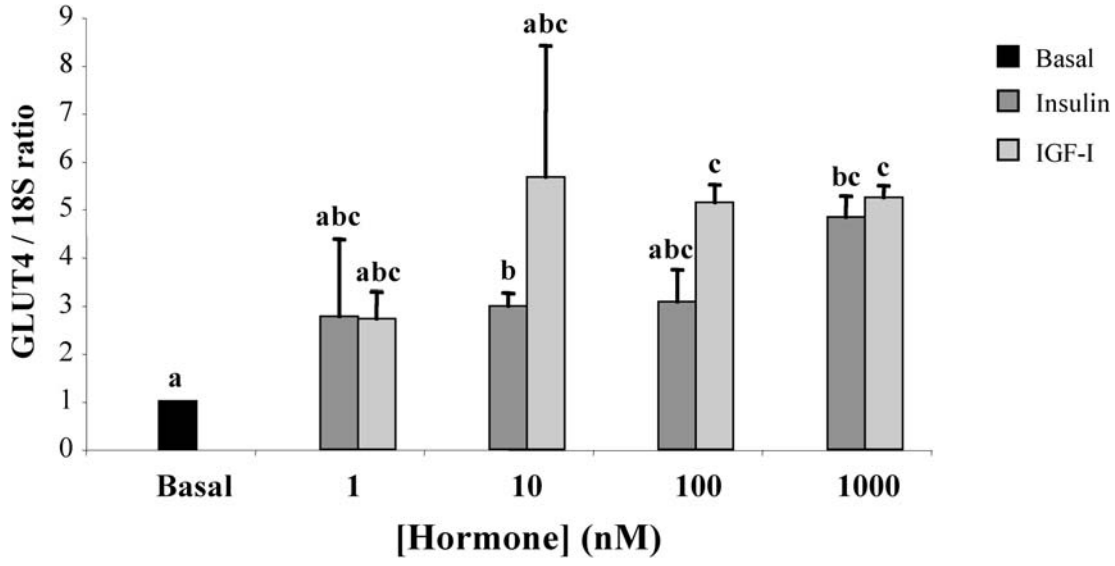

Fig. 7. GLUT4 expression in response to different doses of insulin and IGF-I. After 5 days of culture, muscle cells were serum-starved for 4 h following an 18 h incubation with increasing hormone concentrations (salmon insulin or trout IGF-I) and subsequently processed as described in Materials and methods section. Results are mean ± SE of two independent experiments and were set to 1 in the basal group. Different letters indicate significant differences (P<0.05).

GLUT1 mRNA levels were higher in cells treated with insulin as well as with IGF-I (Fig. 8). In the case of insulin treatment, a clear effect on GLUT1 expression was observed at 10 nM and further increased at the 1000 nM dosage. IGF-I also provoked a significant increase of GLUT1 mRNA at 10 nM, although it appeared to be not significant at the other concentrations tested.

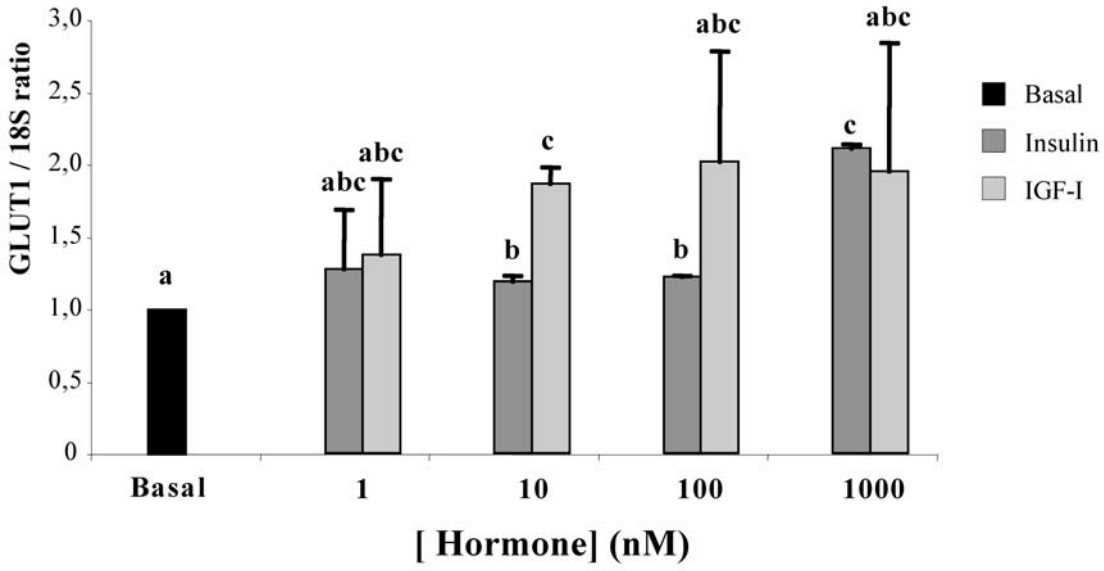

Fig. 8. GLUT1 expression in response to different doses of insulin and IGF-I. After 5 days of culture, muscle cells were serum-starved for 4 h following an 18 h incubation with increasing hormone concentrations (salmon insulin or trout IGF-I) and subsequently processed as described in Materials and methods section. Results are mean ± SE of two independent experiments and were set to 1 in the basal group. Different letters indicate significant differences (P<0.05).

Insulin and IGF-I effects on GLUT4 and GLUT1 expression during 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, myoblast stage) and at day 10 (differentiated cells, myotube stage). Insulin caused a slight increase, although not significant, of GLUT4 mRNA

levels at day 2 and a significant increase at day 10 (Fig. 9A). Similarly, IGF-I provoked changes in GLUT4 expression in trout muscle cells at different stages of differentiation. The stimulatory effects of IGF-I on GLUT4 mRNA levels were significant at day 10, but they could not be evaluated at day 2 due to the non-detectable expression of GLUT4 in the basal state (Fig. 9B). It should be noted that in all cultures the basal expression of GLUT4 was lower at day 2 than at day 10, as observed previously.

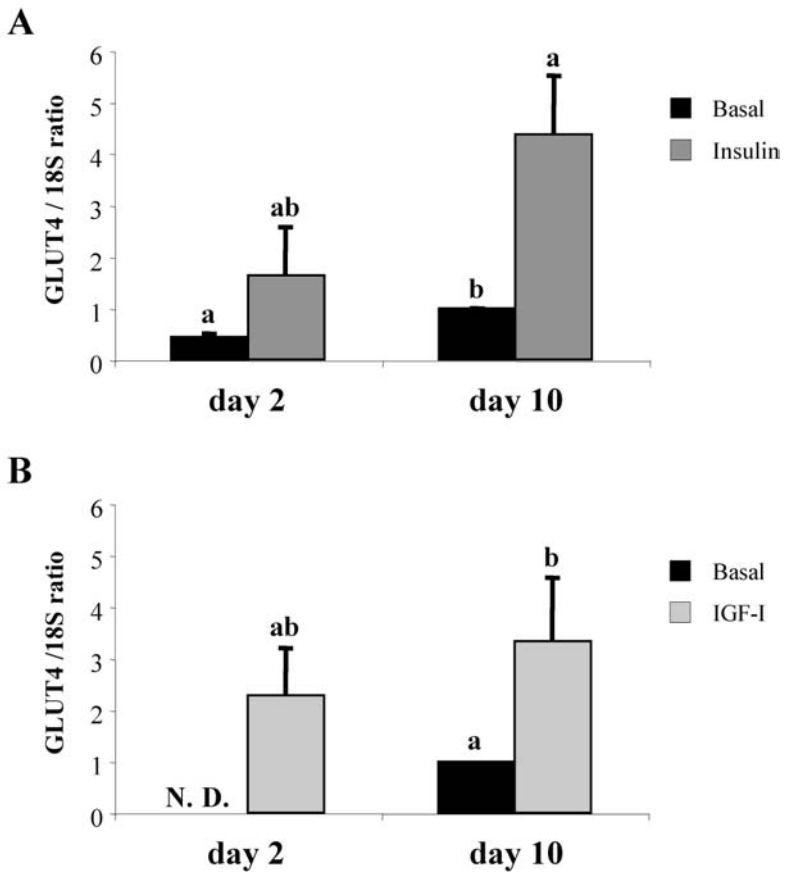

Fig. 9. Insulin (*A*) and IGF-I (*B*) effects on GLUT4 expression at day 2 and 10 of culture. Cells were serum-starved for 4 h and subsequently incubated during 18 h with a medium in the absence or presence of hormone (*A*, 100 nM salmon insulin; *B*, 10 nM trout IGF-I). After the time of incubation, cells were collected and processed as described in Materials and methods section. Results are mean ± SE of two independent experiments referred to basal at day 10, which was set to 1. N. D., non detectable. * $P< 0.05$, ** $P< 0.01$.

Trout muscle cells, in the myoblast and myotube stages, treated with 100 nM insulin for 18 hours did not show a significant increase in GLUT1 mRNA levels over the control cells (Fig. 10A). On the other hand, IGF-I provoked a significant increase in GLUT1 expression in myoblasts, but not in myotubes (Fig. 10B). Furthermore, basal GLUT1 expression was higher in myotubes than in myoblasts.

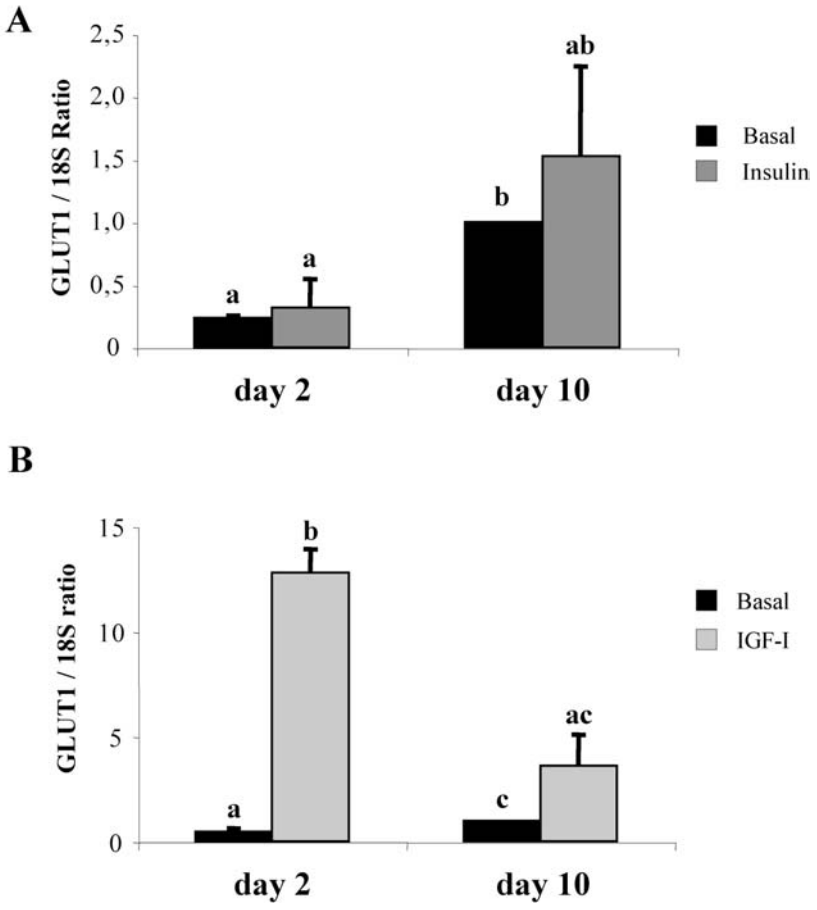

Fig. 10. Insulin (*A*) and IGF-I (*B*) effects on GLUT1 expression at day 2 and 10 of culture. Cells were serum-starved for 4 h and subsequently incubated during 18 h with a medium in the absence or presence of hormone (A, 100 nM salmon insulin; B, 10 nM trout IGF-I). After the time of incubation, cells were collected and processed as described in Materials and methods section. Results are mean ± SE referred to basal at day 10, which was set to 1. *A*, Results from two independent experiments; *B*, Results from three independent experiments. * $P< 0.01$.

DISCUSSION

In the present study, we examined GLUT4 and GLUT1 expression throughout muscle differentiation in trout as well as in response to insulin and IGF-I. For this purpose we used an *in vitro* approach, which consisted of a primary culture of trout muscle satellite cells. This type of culture has been previously well characterized in teleost fishes (Castillo et al., 2002; Fauconneau and Paboeuf, 2000; Koumans et al., 1990; Powell et al., 1989; Rescan et al., 1995) and provides an useful tool to reproduce the muscle differentiation process *in vitro* and to study the function of fish muscle cells.

GLUT4 gene expression was assessed throughout the development of trout myosatellite cells in culture. We have observed that the amount of GLUT4 mRNA gradually increased throughout the differentiation process from myoblasts to myotubes. These data are in agreement with previous studies on human myosatellite cells in culture (Al-Khalili et al., 2003) that demonstrate that GLUT4 expression is higher in myotubes than in myoblasts. In the same way, Guillet-Deniau et al. (1994) 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 mammalian muscle cell line C2C12 (Shimokawa et al., 1998). Furthermore, GLUT4 expression dramatically decreases with de-differentiation of adult rat cardiomyocytes (Rosenblatt-Velin et al., 2004). For this reason the GLUT4 gene is often used as a marker of muscle differentiation. In our study, the increase in GLUT4 expression during trout myoblast differentiation was found to be consistent among the different experiments. However, differences were observed in the magnitude of the increase of GLUT4 expression among different cultures, probably due to the intrinsic variation inherent in primary cultures. Trout muscle satellite cells cultured on a laminin substrate spontaneously differentiate into myotubes without having to reduce the percentage of serum in the media (Rescan et al., 1995). For this reason it is difficult to establish an exact time course of differentiation and it is possible that the speed of differentiation varied somewhat among the different cultures. Furthermore, the higher expression of GLUT4 in trout myotubes may be correlated with an increase in basal glucose uptake of these cells, but this assumption remains to be demonstrated.

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. Our results indicate

that GLUT4 expression is stimulated by insulin and IGF-I in trout muscle satellite cells. Several *in vitro* studies have been conducted in mammals to determine the role of insulin and IGF-I in the regulation of GLUT4 expression. In a primary culture of human muscle satellite cells, a chronic exposure with insulin leads to an increase on GLUT4 mRNA and protein content (Al-Khalili et al., 2005). Similar results are found in rat cardiomyocytes (Petersen et al., 1995). Likewise, GLUT4 expression is stimulated by insulin and IGF-I in fetal brown adipocytes (Valverde et al., 1999). Conversely, studies in muscle and adipogenic cell lines such as L6 or 3T3-L1 cells have reported a down-regulatory effect of insulin and IGF-I on GLUT4 gene expression (Flores-Riveros et al., 1993; Koivisto et al., 1991). Thus, our results agree with the observations made in mammalian primary cultures. In addition, the *in vitro* stimulatory effects of insulin on GLUT4 mRNA levels in trout myosatellite cells are consistent with previous data collected from *in vivo* experiments in trout skeletal muscle. Our group has recently demonstrated that blood insulin levels regulate GLUT4 mRNA expression in trout skeletal muscle (Capilla et al., 2002). Therefore, the present study provides the first evidence that insulin is regulating the amount of GLUT4 mRNA in skeletal muscle by exerting its function directly on trout muscle cells. Furthermore, the induction of GLUT4 expression by insulin required at least 18 hours of incubation with the hormone to be detected, suggesting that insulin may increase GLUT4 mRNA levels in trout muscle cells by increasing the transcriptional activity of the GLUT4 gene. In studies conducted in rat cardiomyocytes and 3T3-L1 adipocytes the effects of insulin on GLUT4 transcription are already evident after 3 hours of treatment (Flores-Riveros et al., 1993; Petersen et al., 1995). In our study, we could notice an increase on GLUT4 expression as early as 6 hours of incubation with insulin, although it was not significant until 18 hours. This correlates with that observed previously in *in vivo* experiments, where GLUT4 mRNA levels in trout red muscle increased 24 hours after insulin treatment (Capilla et al., 2002).

In addition, the regulation of GLUT4 expression by insulin and IGF-I appears to be dependent on the differentiation state of trout muscle cells. Insulin caused a significant increase in GLUT4 mRNA levels in myotubes but this increase was not significant in myoblasts. One possible explanation for the difference in the effects of insulin and IGF-I on GLUT4 expression between myotubes and myoblasts is that insulin action could be mediated, in part, through the IGF-I receptor. In trout muscle cells in primary culture, binding for both insulin and IGF-I has been detected; however,

the number of insulin receptors is very low compared to the number of IGF-I receptors. Furthermore, IGF-I binding gradually increases during myogenesis (Castillo et al., 2002). Insulin, at 100 nM, could bind to both insulin and IGF-I receptors and exert its effect on GLUT4 expression. This is consistent with the fact that, with a ten times lower concentration, IGF-I achieved the same effect on GLUT4 expression in myotubes.

GLUT1 is the other major GLUT isoform in skeletal muscle together with GLUT4 (Klip and Paquet, 1990). We also investigated GLUT1 expression in trout muscle satellite cells and its regulation by insulin. We found that GLUT1 mRNA levels throughout *in vitro* muscle differentiation were more stable than those of GLUT4. In contrast, the myogenic process in mammals is typically characterized by a reduction in GLUT1 expression (Al-Khalili et al., 2003; Guillet-Deniau et al., 1994; Shimokawa et al., 1998; Vinals et al., 1997). Our results suggest that insulin stimulates GLUT1 expression as shown by the time course and dose response experiments performed on day-5 myocytes. However, it is not clear if the insulin effect on GLUT1 expression is related with the myogenic differentiation process, since insulin did not significantly affect the amount of GLUT1 mRNA neither at day 2 nor at day 10 in trout muscle cells in culture. Interestingly, this result is different from the results of a previous *in vivo* study in which we reported that GLUT1 expression in skeletal muscle in trout was not affected by changes in the circulating levels of insulin (Capilla et al., 2002). It is possible that other factors, in addition to insulin, are involved in the *in vivo* regulation of GLUT1 expression and maybe are interfering with the insulin action, suggesting that the regulation of GLUT1 expression could be a complex mechanism. In mammals, it is not clear either if insulin directly regulates GLUT1 expression because different results have been obtained depending on the type of culture used. Studies made with primary cultures of adipocytes and myosatellite cells show that GLUT1 expression does not change in response to an insulin treatment (Al-Khalili et al., 2005; Guillet-Deniau et al., 1994; Hernandez et al., 2003). In contrast, insulin increases the amount of GLUT1 mRNA in 3T3-L1 adipocytes and L6 cells (Garcia de Herreros and Birnbaum, 1989; Taha et al., 1999; Walker et al., 1989). Interestingly, in our study IGF-I caused a drastic increase of GLUT1 expression at day 2 but this increase was not significant at day 10. This feature could have a physiological importance since IGF-I also stimulates proliferation of trout myoblasts (Castillo et al., 2004), being necessary a greater fuel intake to face the increased energy demand. Further studies should be done to address this aspect.

The exact mechanism used by insulin to increase the amount of GLUT4 mRNA in trout muscle cells remains unknown. This increment on GLUT4 mRNA levels could be due to an increased transcription rate of the GLUT4 gene or an increased stability of the transcript. In mammals transcription rate has been suggested for the control of GLUT4 gene expression by insulin (Petersen et al., 1995). In the case of trout GLUT4, the increase of expression caused by insulin may be due to a regulation at the transcriptional level. In support of this idea, our group has been found several putative binding sites for different transcription factors in the sequence of GLUT4 promoter from *Fugu rubripes*. Specifically, we have found two putative binding sites for MEF2, along with other putative binding sites for MyoD and C/EBP (Morata and Planas, unpublished data). These sequence elements have been identified in the mammalian GLUT4 gene promoter and have been shown necessary for the tissue-specific expression of GLUT4. In particular, C/EBP binding site is required for the expression of GLUT4 in adipose tissue (Ezaki, 1997). In a similar manner, the myocyte enhancer factor 2 (MEF2) binding site has been described as essential for GLUT4 expression in skeletal muscle and adipose tissue of mammals (Thai et al., 1998). *In vivo* studies in transgenic mice have demonstrated that MEF2 DNA binding activity is reduced in nuclear extracts from heart and skeletal muscle of diabetic mice, leading to a reduced GLUT4 gene transcription rate. This MEF2 DNA binding activity is recovered after an insulin treatment restoring GLUT4 expression (Thai et al., 1998). In the same way, Santalucía et al. (2001) have reported the cooperation between the transcription factors MyoD, MEF2 and TRγ 1 (thyroid hormone receptor) inducing GLUT4 gene transcription in skeletal muscle cells. In addition, studies on fetal brown adipocytes from rat demonstrate that insulin induces GLUT4 gene expression increasing MEF2 DNA binding activity (Hernandez et al., 2003). Therefore, the insulin effects on GLUT4 mRNA accumulation in trout muscle cells could be mediated by the regulation of these transcription factors, but further investigation will be needed to answer this question.

In summary, our results indicate that insulin regulates GLUT4 expression in fish skeletal muscle. This work provides the first evidence that insulin stimulates GLUT4 expression in skeletal muscle of lower vertebrates by acting directly on muscle cells. This enhanced expression of the GLUT4 gene by insulin will probably lead to an increase of functional GLUT4 molecules in muscle cells, thus improving their ability to take up glucose. In addition, trout GLUT4 expression is induced during myogenesis as its mammalian counterpart, indicating that GLUT4 is also important for fish muscle

development. These results indicate that the mechanisms regulating the expression of GLUT4 have been remarkably well conserved from fish to mammals, evidencing the importance of the maintenance of glucose homeostasis throughout vertebrates.

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CAPÍTOL III: REGULACIÓ DEL TRÀFIC DEL GLUT4 DE TRUITA PER LA INSULINA EN CÈL·LULES MUSCULARS L6

REGULACIÓ DEL TRÀFIC DEL GLUT4 DE TRUITA PER LA INSULINA EN CÈL·LULES MUSCULARS L6

Resum

En mamífers el GLUT4 juga un paper important en l'homeòstasi de la glucosa mediant l'acció de la insulina per augmentar la captació de glucosa en aquells teixits sensibles a la insulina. En estat basal el GLUT4 es troba localitzat en compartiments intracel·lulars i en resposta a la insulina és reclutat cap a la membrana plasmàtica on permet l'entrada de glucosa dins la cèl·lula. Recentment el nostre grup ha identificat un homòleg del GLUT4 en el múscul esquelètic de truita comuna (btGLUT4). En aquest treball aportem les primeres evidències en la regulació del tràfic d'aquest transportador per la insulina. Per tal d'assolir aquest objectiu, es va insertar l'epítop myc a la seqüència del btGLUT4 (btGLUT4myc), i el btGLUT4myc va ser expressat en cèl·lules L6. A continuació, es van investigar els efectes de la insulina sobre la translocació del btGLUT4myc, la captació de glucosa i la internalització del btGLUT4myc en aquestes cèl·lules. La insulina va estimular la translocació del btGLUT4myc en les cèl·lules L6-btGLUT4myc però en menor grau que el GLUT4myc de rata. A més, en estat basal un percentatge més elevat de btGLUT4myc es trobava insertat a la membrana plasmàtica comparat amb el GLUT4myc de rata. Així mateix, l'endocitosi del btGLUT4myc era més lenta que l'observada en el GLUT4myc de rata. Tanmateix, la insulina va estimular la captació de glucosa en les cèl·lules L6 que expressaven el btGLUT4myc de manera dosi-dependent, així com a la línia cel·lular que sobreexpressa el GLUT4 de rata. Tots aquests resultats suggereixen que les diferències observades en el tràfic del GLUT4 de truita i el del GLUT4 de rata poden estar relacionades amb diferències en la seqüència d'alguns motius els quals s'ha demostrat que són importants pel tràfic intracel·lular del GLUT4 de mamífer.

Endocrinology (in preparation)

REGULATION OF FISH GLUT4 TRAFFIC BY INSULIN IN L6 MUSCLE CELLS

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Running title: Insulin action on fish GLUT4 traffic

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ABSTRACT

In mammals GLUT4 plays an important role in glucose homeostasis mediating insulin action to increase glucose uptake in insulin-responsive tissues. In the basal state GLUT4 is located in intracellular compartments and upon insulin stimulation is recruited to the plasma membrane where it allows the entrance of glucose into the cell. Recently, our group identified a GLUT4-homolog in skeletal muscle from brown trout (btGLUT4). Here, we report our initial findings on the traffic regulation of this glucose transporter by insulin. To address this issue btGLUT4 tagged with the myc epitope (btGLUT4myc) was stably overexpressed in L6 muscle cells and insulin effects on btGLUT4myc translocation, glucose uptake as well as btGLUT4myc internalization in these cells were examined. Insulin stimulated btGLUT4myc translocation in L6-btGLUT4myc cells but to a lesser extent than rat GLUT4myc. Furthermore, in the basal state a higher percentage of btGLUT4myc was found to be residing at the plasma membrane compared to ratGLUT4myc. Likewise, btGLUT4myc endocytosis was slower than that observed for rat GLUT4myc. Nevertheless, insulin stimulated glucose uptake in btGLUT4myc-expressing L6 cells in a dose dependent manner, as well as in the rat cell line. Taking together these data suggest that differences observed between brown trout GLUT4 and rat GLUT4 in terms of traffic may be related to differences in the sequence of some motifs shown to be important for the intracellular targeting of mammalian GLUT4.

INTRODUCTION

In mammals, GLUT4 is the main glucose transporter expressed in insulin-sensitive tissues such as adipose tissue, skeletal muscle and heart (Scheepers et al., 2004; Wood and Trayhurn, 2003). This facilitative glucose transporter exerts its function at the plasma membrane allowing the glucose entrance into muscle and fat cells. It has been demonstrated that in the basal state, most of the amount of GLUT4 is located in intracellular stores and that in response to insulin is rapidly translocated to the cell surface resulting in an increase in cellular glucose transport (Bryant et al., 2002; Watson and Pessin, 2006). For this reason GLUT4 has been described as the insulin-responsive glucose transporter, and it is important for the rapid glucose disposal from blood in a situation of increased glucose plasma levels, such as during the postprandial period. Martin et al. (2006) have recently proposed that in the basal state, GLUT4 retention in intracellular storage sites is a dynamic process, based on a slow exocytosis and a rapid internalization from the plasma membrane. Thus, insulin action focuses on accelerate exocytosis (Foster et al., 2001; Yang et al., 1996) and reduce the internalization rate of mammalian GLUT4 (Czech and Buxton, 1993). Following biosynthesis GLUT4 is targeted from the trans-Golgi network (TGN) to specific membrane compartments that are insulin-responsive. It has been demonstrated that this initial sorting decision is dependent on the GGA (Golgi-localized, γ-ear-containing, Arf-binding protein) adaptor complex (Li and Kandror, 2005; Watson et al., 2004b). Likewise, GLUT4 contains intrinsic targeting signals that modulate its intracellular retention in this insulin-responsive compartment. In particular, a dileucine motif and an acidic sequence located at the carboxy terminus have been involved in this process (Martinez-Arca et al., 2000). Furthermore, the dileucine signal has also been implicated in the internalization of GLUT4 from the plasma membrane (Corvera et al., 1994; Verhey et al., 1995). Several studies have examined the elements involved in the intracellular sequestration of GLUT4 in adipocytes. Bogan et al. (2003) have reported that TUG (tether, containing a UBX domain, for GLUT4) protein binds GLUT4 and retains it in the specialized compartment in absence of insulin stimulation. Recently, some authors have shown that AS160 Rab GAP is essential for the basal intracellular retention of GLUT4 in 3T3-L1 adipocytes (Eguez et al., 2005). This protein is associated with the GLUT4 vesicles in the basal state and dissociates in response to

insulin. Therefore, reduced expression of AS160 causes an increase on plasma membrane levels of mammalian GLUT4 in the basal state (Larance et al., 2005).

Our group has identified, for the first time in non-mammalian vertebrates, two GLUT4-homologs in fish, one in brown trout skeletal muscle (btGLUT4) and another one in salmon adipose tissue (okGLUT4) (Capilla et al., 2004; Planas et al., 2000a). Kinetic studies of okGLUT4 expressed in *Xenopus* oocytes have shown that it transports glucose across the cell membrane but with a lower affinity than mammalian GLUT4 (Capilla et al., 2004). This difference in affinity may explain the lower ability of fish to clear a glucose load, when compare to mammals (Blasco et al., 1996; Moon, 2001). Furthermore, okGLUT4 is able to translocate to the plasma membrane in response to insulin when it is transiently expressed in 3T3-L1 adipocytes (Capilla et al., 2004). In fish, the major site for glucose uptake is the skeletal muscle since it represents more than 50% of fish body weight (Blasco et al., 1996). Following our interest in studying the function of GLUT4 in fish skeletal muscle we initially studied the physiological regulation of GLUT4 expression in red and white muscle. We have previously shown that btGLUT4 expression is regulated by blood insulin levels in red, but not in white, skeletal muscle (Capilla et al., 2002). Furthermore, we have also shown that insulin directly stimulates glucose uptake as well as GLUT4 expression in a primary culture of trout muscle satellite cells (Díaz and Planas, unpublished; see chapters II and IV). In the same way, Castillo et al. (2004) recently reported a rapid effect of insulin and IGF-I increasing glucose uptake in rainbow trout muscle cells. This short-term effect of insulin may be presumably due to a post-translational regulation of glucose transporters.

However, we do not know whether btGLUT4 traffic in muscle cells is regulated by insulin. Rat L6 cell line is a suitable model that has been extensively used to study GLUT4 traffic in skeletal muscle (Rudich and Klip, 2003). In order to characterize the regulation of btGLUT4 translocation in muscle cells and due to the lack of a fish muscle cell line, we have generated a L6 cell line that stably overexpresses btGLUT4 harbouring an exofacial myc epitope (btGLUT4myc). Our initial results indicate that btGLUT4myc is increased at the cell surface of L6 cells in response to insulin, but to a lesser extent than rat GLUT4myc. In addition, the basal rate of internalization of btGLUT4myc appears to be slower than that observed in rat GLUT4myc L6 cells. These data suggest that translocation of btGLUT4 is regulated by insulin in muscle cells but to a lesser extent than its mammalian counterpart. Therefore, we speculate that the

difference between fish and mammalian GLUT4 in terms of membrane trafficking could be related to differences in the sequence of certain protein motifs shown to be important for internalization and intracellular retention of GLUT4.

MATERIAL AND METHODS

Materials

γ-MEM, FBS and all other tissue culture reagents were purchased from Invitrogen (Prat del Llobregat, Spain). Plasticware for cell culture was from BD Biosciences (Madrid, Spain). The Effectene Transfection Reagent was from QIAGEN (Hilden, Germany). Human insulin (Humulin R) was from Lilly (Alcobendas, Madrid). O-phenylenediamine dihydrochloride (OPD) reagent was from Sigma (Tres Cantos, Madrid). Monoclonal and polyclonal anti-myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from Jackson Immunoresearch (Soham, United Kingdom). 2-Deoxy-D-[2,6-^{3}H]glucose was purchased from Amersham Biosciences (Barcelona, Spain). Bio-Rad Protein Assay was obtained from Bio-Rad (Prat del Llobregat, Spain).

Construction of c-myc epitope-tagged btGLUT4 cDNA expression vector

The cDNA sequence of human c-myc epitope (GCAGAGGAGCAAAAGCTTATTTCTGAAGAGGACTTGCTTAAG) was introduced into the cDNA coding sequence of brown trout GLUT4 (btGLUT4), between Gly^{58} and Glu^{59}, in the region corresponding to the first extracellular loop of btGLUT4 protein (Fig. 1A). This insertion was carried out by the overlapping PCR method. Two rounds of PCR were necessary to obtain the final construct. In the first round, two DNA fragments were obtained separately: one fragment corresponding to the btGLUT4 cDNA sequence coding for the amino acids 1-58 and carrying the cDNA sequence of c-myc epitope at the 3' end and another DNA fragment corresponding to the btGLUT4 cDNA sequence coding for the amino acids 59-503 and carrying the c-myc sequence at the 5' end. At the second round of PCR these two initial fragments were used as templates to obtain the cDNA coding sequence of btGLUT4 with the

inserted sequence of myc (btGLUT4myc). Finally btGLUT4myc was subcloned into the mammalian expression vector pCXN2.

Generation of L6-btGLUT4myc stable cell line

Parental L6 myoblasts were cotransfected with the expression vector pCXN2-btGLUT4myc and the pSV2-bsr plasmid (a blasticidin S deaminase expression plasmid) using the Effectene Transfection Reagent. Transfected cells were selected with blasticidin S hydrochloride. Several clones were isolated and their btGLUT4myc expression and ability to fusion were analyzed.

Cell culture

L6 myoblasts were maintained with γ-MEM containing 10% FBS and 1% antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, 25 μg/ml amphotericin B) in an atmosphere of 5% CO_2 at 37°C. For GLUT4myc-expressing L6 cells the medium was also supplemented with 2 μg/ml blasticidin hydrochloride. To induce differentiation of myoblasts into myotubes the percentage of FBS in the medium was reduced to 2%. For the experiments carried out with myoblasts, cells were used at confluence two days after seeding. For the experiments with myotubes, cells were used seven days after seeding. In all the experiments except for immunoprecipitation cells were seeded in 24-well plates. Prior to the incubations with insulin or sucrose the cells were serum-depleted for 3-5 hours.

Immunoprecipitation and immunoblotting of GLUT4myc

L6 myoblasts grown on six-well plates were lysed on ice with PBS containing 0.2% octaethylene glycol monododecyl ether ($C_{12}E_8$) and a protease inhibitor cocktail (104 μM AEBSF, 80 nM aprotinin, 2 μM leupeptin, 4 μM bestatin, 1.5 μM pepstatin A and 1.4 μM E-64). Lysates were passed 5 times through a 25-gauge syringe and centrifuged at 12000 x g for 5 min at 4°C. Supernatants were collected and analyzed for protein concentration with the Bio-Rad Protein Assay. For each sample 300 μg of protein were incubated with 2 μg of monoclonal (9E10) anti-myc antibody overnight at 4°C with agitation. Immunocomplexes were sedimented with protein G sepharose and eluted with 2 x Laemmli sample buffer. Subsequently, immunocomplexes were

resolved by 10% SDS-PAGE, transferred to a PVDF membrane and immunoblotted with polyclonal (A-14) anti-myc antibody at 1:1000 dilution.

GLUT4myc translocation assay

Detection of cell surface GLUT4myc was carried out by a colorimetric assay described by Li et al. (2001) with slight modifications. L6 cells were grown until confluence and were serum-starved for 3-5 h prior to the incubation in the absence or presence of 100 nM insulin or 0.45 M sucrose for 20 min at 37^{o}C. After the stimulation period plates were placed on ice and cells were washed with ice-cold PBS. After blocking with 5% goat serum in PBS for 10 min cells were incubated with polyclonal anti-myc antibody (2 μg/ml in PBS containing 5% goat serum) for 90 min at 4^{o}C. After several washes with PBS cells were fixed with 3% paraformaldehyde in PBS for 10 min at 4^{o}C. Subsequently, cells were quenched with 100 mM glycine in PBS for 10 min following the incubation with HRP-conjugated goat anti-rabbit IgG (1:1000 in PBS containing 5% goat serum) for 1 h at 4^{o}C. After six washes with PBS cells were incubated with OPD reagent (the HRP substrate) for 5-10 min at room temperature and protected from light. The reaction was stopped by the addition of 3 N HCl. An aliquot of each well was transferred to a 96-well plate and the optical absorbance was measured at 492 nm. Total GLUT4myc content was determined by permeabilizing the cells with 0.1% triton X-100 for 20 min prior to incubation with the antibody against myc.

2-Deoxyglucose uptake measurement

Determination of 2-deoxyglucose uptake was performed as described by Huang et al. (2005) with some modifications. Glucose uptake was assessed for 5 min in HEPES-buffered saline (140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4) containing 10 μM 2-deoxyglucose (0.5 μCi/ml 2-[^{3}H]-deoxyglucose) at room temperature. Subsequently, cells were rinsed three times with an ice-cold solution containing 0.9% NaCl and 20 mM D-glucose. To quantify the radioactivity incorporated by the cell, cells were lysed with 0.05 N NaOH and lysates were counted with scintillation liquid in a γ-counter. Nonspecific uptake was carried out in the presence of cytochalasin B during the assay, and these values were subtracted from all other values. The concentration of cytochalasin B used ranged from 20 to 50 μM. Total protein content was measured with the Bio-Rad Protein Assay.

GLUT4myc internalization assay

L6 myoblasts were serum starved for 3-5 h and incubated in the absence or presence of 100 nM insulin for 20 min at 37°C. After labeling cell surface GLUT4myc with the polyclonal anti-myc antibody for 1 h at 4°C, cells were washed with PBS and incubated with pre-warmed γ -MEM containing or not 100 nM insulin during 2 min at 37°C to allow GLUT4myc endocytosis. The myc antibody-labeled GLUT4myc remaining at the cell surface was measured with the colorimetric assay mentioned above and the results were expressed as a percentage of cell surface GLUT4myc level at 0 min of endocytosis.

Statistical analysis

Values are given as mean ± SE. Data were analyzed using SPSS software. Differences between groups were evaluated by one-way ANOVA and Student's t-test.

RESULTS

Expression analysis of btGLUT4myc in the isolated clones

L6 cells were cotransfected with pCXN2-btGLUT4myc and pSV-bsr plasmids. Cell clones resistant to blasticidin hydrochloride were assessed for btGLUT4myc expression. Only five of the 17 clones tested were positive for btGLUT4myc expression (Fig. 1B). The L6 cell clone with highest btGLUT4myc expression was the clone #6, followed by clones #9 and #15. Clones #7 and #14 had the lowest levels of expression of btGLUT4myc. Only clone #6 had GLUT4myc expression comparable to the L6 cell line that overexpresses rat GLUT4 tagged with the myc epitope (L6-ratGLUT4myc) (Ueyama et al., 1999). According to the ability of the different clones to fuse and form myotubes clones #6, #7 and #9 were chosen for subsequent studies.

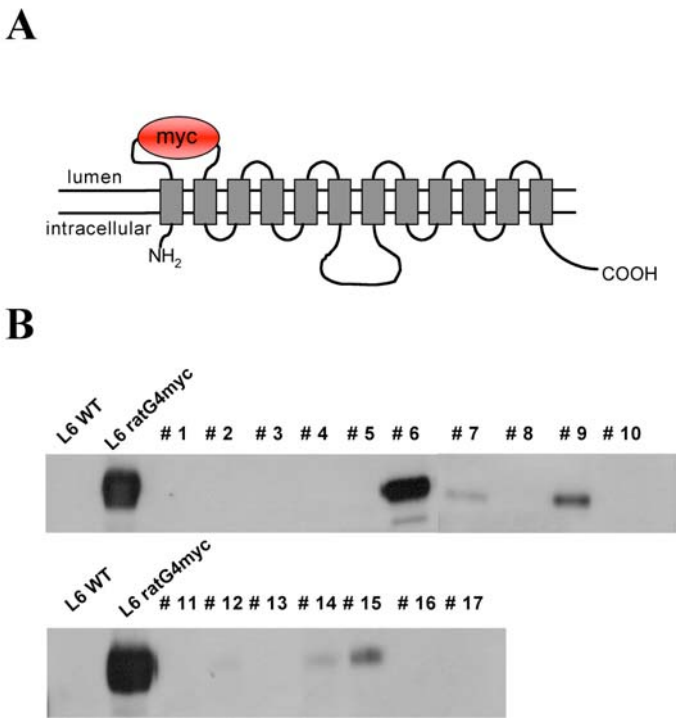

Fig. 1. *A*, Scheme of the structure of btGLUT4myc. Both amino and carboxy tails are cytoplasmic and the myc epitope is inserted at the first extracellular loop. *B*, L6 myoblasts were co-transfected with the btGLUT4myc cDNA and the blasticidin-resistance cDNA and were selected with blasticidin S hydrochloride. Several clones were isolated and its btGLUT4myc expression was analyzed. Total protein extracts from cell lines (300 μg) were immunoprecipitated using anti-myc monoclonal antibody. Immunocomplexes were resolved by 10% SDS-PAGE and analyzed by Western blotting using a polyclonal antibody against the myc epitope.

GLUT4myc translocation in response to insulin and hypertonicity

The amount of GLUT4myc present at the cell surface was measured in the different L6 clones that overexpress btGLUT4myc (L6-btGLUT4myc) after a 20 min treatment with 100 nM insulin or 0.45 M sucrose (hypertonic condition). In parallel the same determinations were done in L6-ratGLUT4myc cell line in order to compare the response between the rat and the fish clones. Insulin caused an increase of btGLUT4myc at the plasma membrane in all the L6 cell lines expressing btGLUT4 in the myoblast stage, as well as in the L6 ratGLUT4myc cell line (Fig. 2A). The net gain of GLUT4myc at the cell surface in response to insulin appeared to be higher in L6-ratGLUT4myc cells (2.05 ± 0.17) than in the L6-btGLUT4myc clones (1.39 ± 0.06, 1.24 ±0.07 and 1.35 ± 0.06 for clones #6, #7 and #9, respectively). Hyperosmolarity

also provoked ratGLUT4myc translocation to the plasma membrane in myoblasts but, surprisingly, did not have any effect on btGLUT4myc translocation in any of the three clones expressing btGLUT4myc. In myotubes, btGLUT4myc levels at the cell surface increased in response to insulin, but this increment was only significant in clone #6. Furthermore, hypertonic sucrose did not cause btGLUT4myc translocation in myotubes. The level of ratGLUT4myc at the cell surface also increased in response to insulin but to a lesser extent than in myoblasts (Fig. 2B). Conversely, sucrose did not increase cell surface ratGLUT4myc in myotubes. Absolute values of translocation in the basal state could not be compared among the different cell lines due to the differences in the expression levels of GLUT4myc.

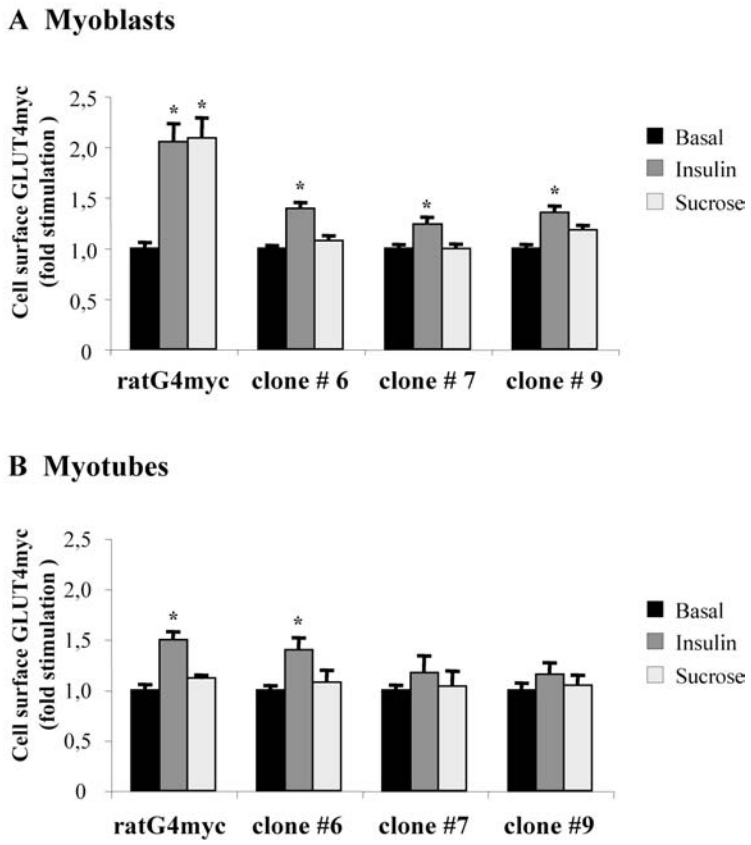

Fig. 2. Steady state levels of GLUT4myc at the cell surface of L6 myoblasts (*A*) and myotubes (*B*) in response to insulin and hyperosmolarity. Cells were serum-deprived for 3-5 h and were subsequently incubated in the absence or presence of 100 nM insulin or 0.45 M sucrose for 20 min at 37°C. After the incubation period cell surface GLUT4myc level was determined as described under "Materials and methods". Results are expressed as fold stimulation above the basal level of each cell line which was set to 1. Data are mean ± SE of three independent experiments each performed in triplicate. * Significant differences compared to basal (P<0.05).

Moreover, when cell surface GLUT4myc is expressed as the percentage of total GLUT4myc, the level of GLUT4myc at the cell surface under basal conditions was significantly higher for L6-btGLUT4myc cells than for L6-ratGLUT4myc cells (41.03 ± 3.72 vs. 29.84 ± 2.18) (Fig. 3). As shown before, insulin, but not sucrose, stimulated the cell surface levels of btGLUT4myc; whereas, both insulin and sucrose increased the levels of cell surface ratGLUT4myc.

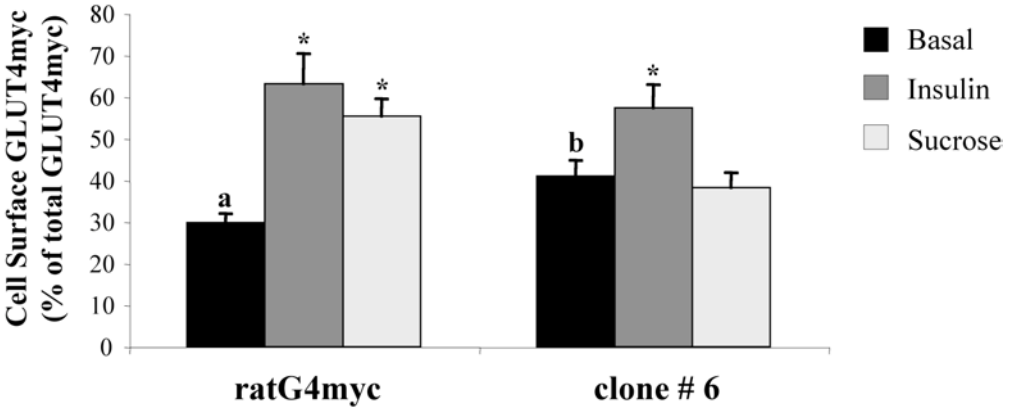

Fig. 3. Steady state levels of GLUT4myc at the cell surface of L6 myoblasts in response to insulin and hyperosmolarity. Cells were serum-deprived for 3-5 h and were subsequently incubated in the absence or presence of 100 nM insulin or 0.45 M sucrose for 20 min at 37°C. After the incubation period cell surface GLUT4myc level and total GLUT4myc were determined as described under "Materials and methods". Results are expressed as the percentage of GLUT4myc inserted at the cell surface. Data are mean ± SE of three independent experiments each performed in triplicate. * Significant differences compared to basal. Different letters indicate significant differences between basal groups (P<0.05).

The lower response of btGLUT4myc to insulin in terms of translocation to the plasma membrane in L6 cells led us to examine the temporal pattern of insulin stimulation by performing a time course experiment. L6 myoblasts overexpressing ratGLUT4myc or btGLUT4myc (clone #6) were incubated in the absence or presence of 100 nM insulin or 0.45 M sucrose for 5, 10, 20 and 40 minutes. After the incubation period GLUT4myc at the cell surface was quantified. Translocation of ratGLUT4myc to the plasma membrane in response to insulin was already visible at 5 minutes and

maintained until 20 minutes, but it was not significant at 40 minutes. Similarly, the effect of sucrose on ratGLUT4myc translocation was clear at 5 minutes and did not vary significantly in the incubation times assessed (Fig. 4A). In the case of btGLUT4myc, the translocation to the cell surface in response to insulin was not statistically significant until 10 minutes, reaching its maximum at 20 minutes and decreasing at 40 minutes until not significant levels. Sucrose did not increase the amount of btGLUT4myc at the plasma membrane at any time assessed, causing even a decrease at 40 minutes (Fig. 4B).

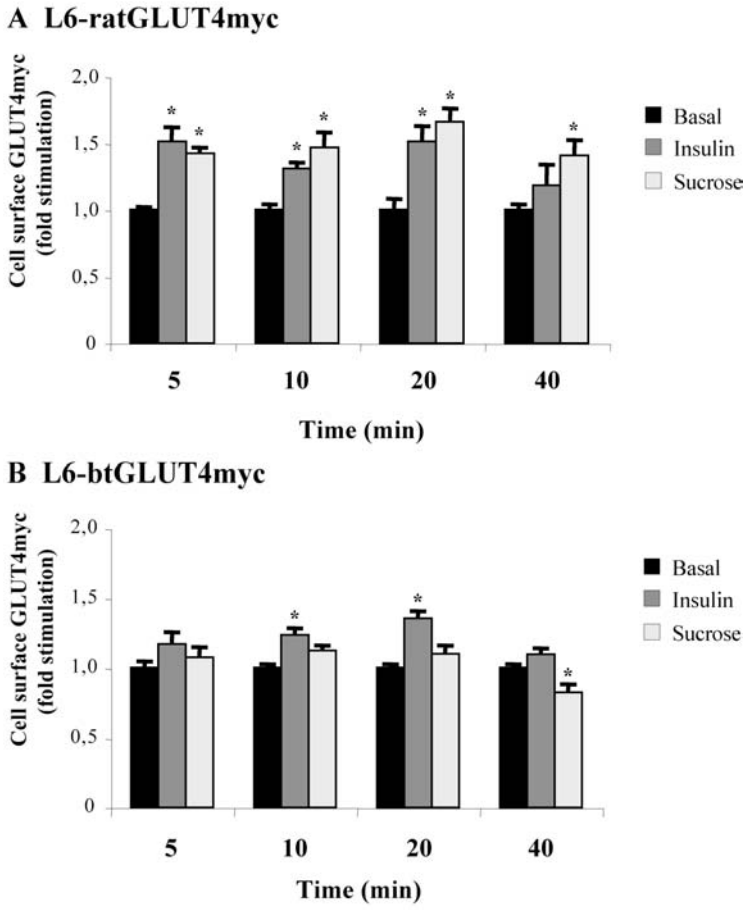

Fig. 4. Time course of insulin and hyperosmolarity effects on steady state levels of cell surface ratGLUT4myc (*A*) and btGLUT4myc (*B*). L6 myoblasts were serum-deprived for 3 h and subsequently incubated for the indicated times with or without 100 nM insulin or 0.45 M sucrose at 37°C. After the incubation period cell surface GLUT4myc level was determined as described under "Materials and methods". Results are expressed as fold stimulation above the basal level of each time which was set to 1. *A*, Basal values of cell surface ratGLUT4myc at 5, 10, 20 and 40 min were 1±0.02, 1.19±0.15, 1.05±0.13, 1.02±0.11, respectively. *B*, Basal values of cell surface btGLUT4myc at 5, 10, 20 and 40 min were 1±0.05, 0.88±0.05, 0.95±0.04 and 1.02±0.06, respectively. Data are mean ± SE of four independent experiments each performed in triplicate. * Significant differences compared to basal of each time ($P<0.05$).

Glucose uptake in response to insulin and hypertonicity

Glucose uptake measurements in the different L6-btGLUT4myc clones were conducted to assess the effect of btGLUT4myc overexpression in cells at the myoblast and myotube stages. After 3-5 hours of serum starvation L6 cells were incubated with different concentrations of insulin (0, 10 and 100 nM) for 20 min followed by the glucose uptake assay. In parallel the same experiments were also performed in wild-type L6 cells (untransfected cells) and L6-ratGLUT4myc cells. In myoblasts, all the btGLUT4myc clones together with wild-type L6 cells and L6-ratGLUT4myc cells presented a similar pattern of insulin-stimulated glucose uptake (Fig. 5A). In all cases insulin already stimulated glucose uptake at 10 nM and this stimulation was slightly higher at 100 nM.

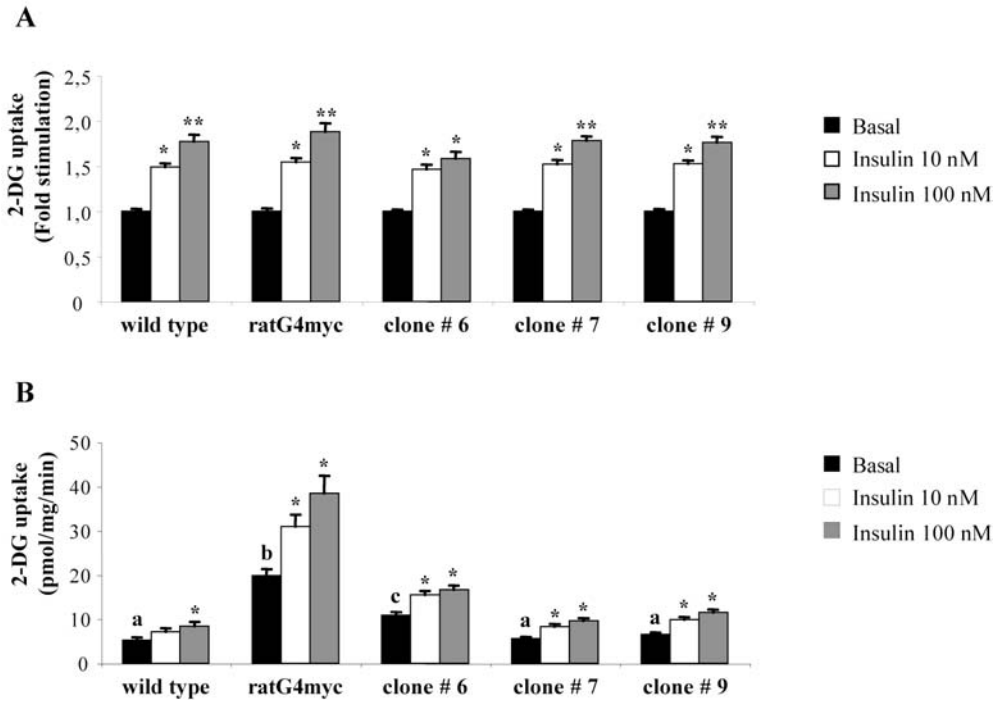

Fig. 5. Dose response of insulin-stimulated 2-deoxyglucose uptake in L6 myoblasts. Cells were serum-deprived for 3-5 h and subsequently incubated with the indicated concentrations of insulin for 20 min at 37°C. After the incubation period 2-deoxyglucose uptake assay was performed as indicated under "Material and methods". *A*, Results are expressed as fold stimulation above basal level of each cell line which was set to 1. *B*, Results are expressed as absolute values of pmoles of 2-DG per mg of protein and per minute. Data are mean ± SE of six independent experiments (except for wild type cells that were four) each performed in triplicate. * Significant differences compared to basal of each cell line. ** Significant differences compared to basal and 10 nM insulin. Different letters indicate significant differences ($P<0.05$).

However, absolute values of glucose uptake expressed as pmoles of 2-deoxyglucose per minute and per mg of protein differed among cell lines (Fig. 5B). L6-ratGLUT4myc cells had the highest glucose uptake rate under basal conditions, in accordance with the level of expression of GLUT4myc, followed by clone #6 of L6-btGLUT4myc cells. Interestingly, glucose uptake in the basal state of clones #7 and #9 did not significantly differ from the basal glucose uptake of untransfected L6 cells.

In myotubes, insulin stimulated glucose uptake in a dose-dependent fashion, in a manner to that observed in myoblasts (Fig. 6A). L6-ratGLUT4myc myotubes showed the highest glucose uptake rate in the basal state compared to the other cell lines (Fig. 6B).

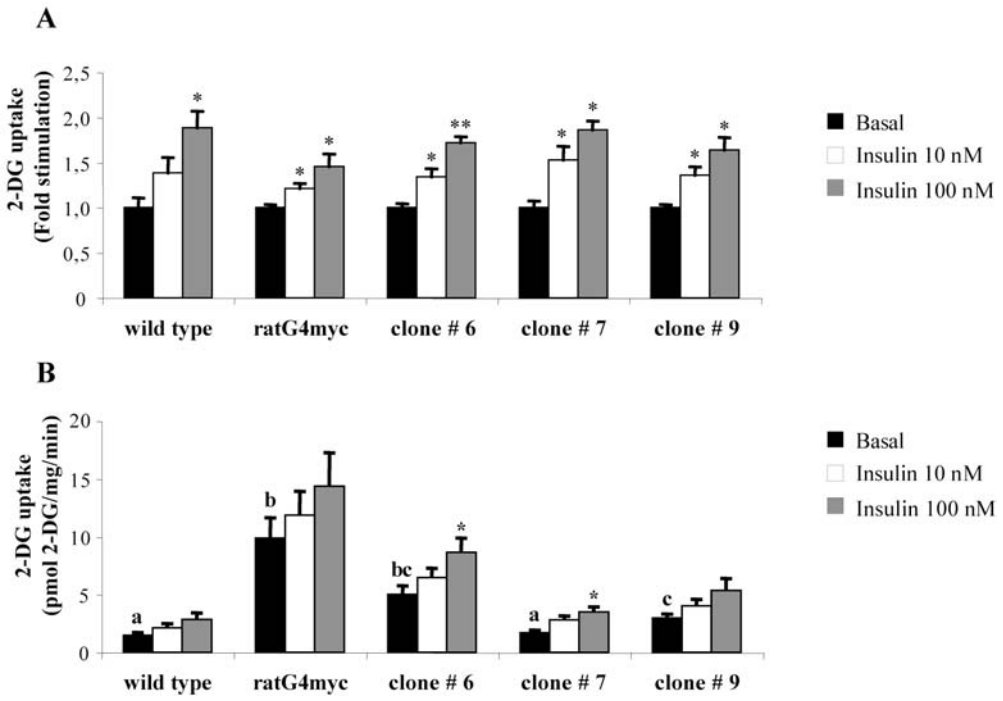

Fig. 6. Dose response of insulin-stimulated 2-deoxyglucose uptake in L6 myotubes. Cells were serum-deprived for 3-5 h and subsequently incubated with the indicated concentrations of insulin for 20 min at 37°C. After the incubation period 2-deoxyglucose uptake assay was performed as indicated under "Material and methods". *A*, Results are expressed as fold stimulation above basal level of each cell line which was set to 1. *B*, Results are expressed as absolute values of pmoles of 2-DG per mg of protein and per minute. Data are mean ± SE of four independent experiments each performed in triplicate. * Significant differences compared to basal of each cell line. ** Significant differences compared to basal and 10 nM insulin. Different letters indicate significant differences (P<0.05).

Basal glucose uptake in myotubes from btGLUT4myc-expressing clones #6 and #9 was significantly higher than that of wild-type myotubes but lower than in those expressing ratGLUT4myc. Conversely, basal glucose uptake in myotubes from clone #7 did not differ from the basal glucose uptake on wild-type L6 myotubes. It should be noted that, for all cell lines, glucose uptake rate in myotubes was always lower than in myoblasts.

The effects of sucrose on 2-deoxyglucose uptake were also investigated in btGLUT4myc-expressing clone #6 and compared with L6-ratGLUT4myc cells. Hypertonic conditions increased glucose uptake in myoblasts from clone #6, but to a lesser extent than in L6-ratGLUT4myc myoblasts, whereas insulin caused a similar increase of glucose uptake in both cell lines (Fig. 7A). In contrast, sucrose did not affect glucose uptake in myotubes from clone #6 significantly (Fig. 7B).

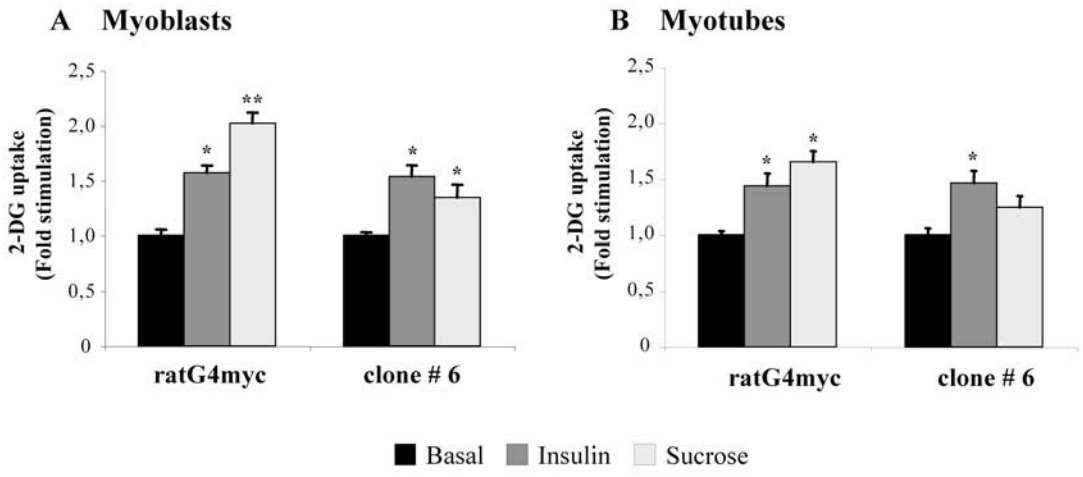

Fig. 7. Effect of insulin and hyperosmolarity on 2-deoxyglucose uptake in L6 myoblasts (*A*) and myotubes (*B*). Cells were serum-depleted for 3-5 h and subsequently incubated with or without 100 nM insulin or 0.45 M sucrose for 20 min at 37°C. After the incubation period 2-deoxyglucose uptake assay was performed as indicated under "Material and methods". Results are expressed as fold stimulation above basal level of each cell line which was set to 1. *A*, Absolute values of basal 2-deoxyglucose uptake for L6-ratGLUT4myc and L6-btGLUT4myc (clone # 6) myoblasts were 11.9 ± 1.1 and 7.6 ± 0.8 pmol 2-DG/mg/min, respectively. Data are mean ± SE of three independent experiments each performed in triplicate. *B*, Absolute values of basal 2-deoxyglucose uptake for L6-ratGLUT4myc and L6-btGLUT4myc (clone # 6) myotubes were 10.5 ± 0.4 and 4.2 ± 0.3 pmol 2-DG/mg/min, respectively. Data are mean ± SE of two independent experiments each performed in triplicate. * Significant differences compared to basal of each cell line. ** Significant differences compared to basal and 100 nM insulin ($P<0.05$).

We also investigated the sensitivity of btGLUT4myc cell line to cytochalasin B, a well-known inhibitor of facilitated glucose transport, and compared it to the ratGLUT4myc cell line (Fig. 8). Glucose uptake was clearly inhibited by cytochalasin B in a concentration dependent manner both on rat and btGLUT4myc cells, but ratGLUT4myc cells were more sensitive to the inhibitor. For example, 1 μM cytochalasin B reduced glucose uptake in L6-ratGLUT4myc myoblasts by 87%, whereas the same concentration of cytochalasin B reduced glucose uptake in L6-btGLUT4myc only to 50% of the initial glucose uptake.

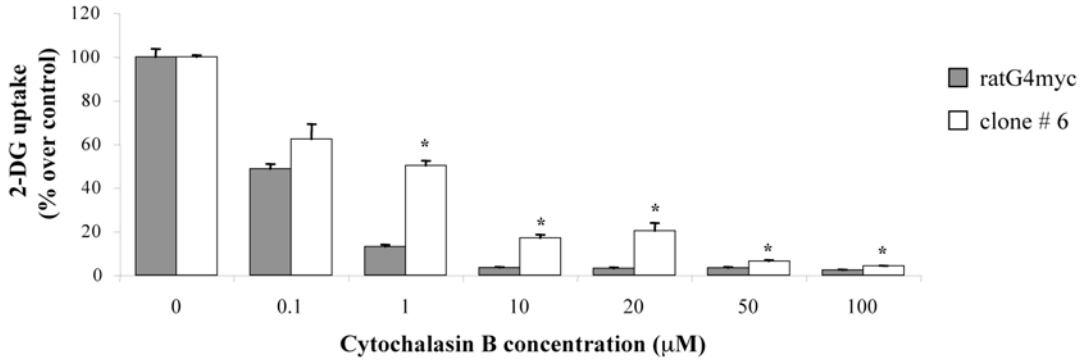

Fig. 8. Inhibition of 2-deoxyglucose uptake in L6-ratGLUT4myc and L6-btGLUT4myc (clone # 6) myoblasts by increasing concentrations of cytochalasin B. 2-deoxyglucose uptake assay was performed in the presence of the indicated concentrations of cytochalasin B in the transport solution. Results are expressed as a percentage of uptake over the basal value obtained without inhibitor. Data are mean ± SE of two independent experiments each performed in triplicate. * Significant differences between cell lines at the same concentration of inhibitor (P<0.05).

GLUT4myc endocytosis in L6 myoblasts

Internalization rates of ratGLUT4myc and btGLUT4myc were analyzed quantifying the amount of myc-tagged transporters remaining at the cell surface after 2 minutes of endocytosis. Under basal conditions the percentage of btGLUT4myc remaining at the cell surface was significantly higher in clones #6 and #7 compared with ratGLUT4myc (Fig. 9A). In addition, insulin did not affect the internalization of btGLUT4myc but prevented, at least in part, ratGLUT4myc endocytosis (Fig. 9B).

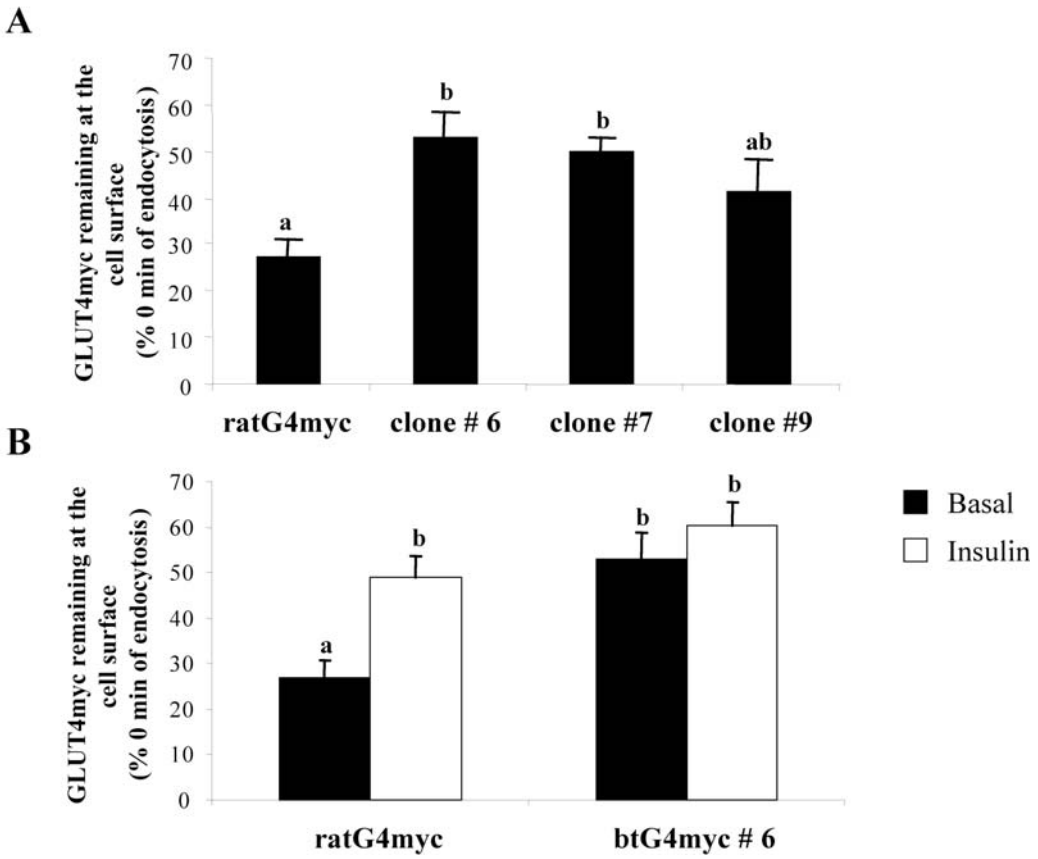

Fig. 9. GLUT4myc internalization. L6 myoblasts from each clone were serum-starved for 3 h and stimulated without or with 100 nM insulin for 20 min at 37°C. After labeling cell surface GLUT4myc with anti-myc antibody for 1 h at 4°C, cells were washed with PBS and incubated with pre-warmed media containing or not 100 nM insulin for 2 min at 37°C to allow endocytosis. GLUT4myc remaining at the cell surface was measured with the OPD optical densitometric assay. *A,* Percentage of GLUT4myc remaining at the cell surface after 2 min of internalization in the basal state. *B*, Effect of insulin on GLUT4myc internalization. Data are the mean ± S.E. of three independent experiments each performed in triplicate. Different letters indicate significant differences at $P<0.05$.

DISCUSSION

In the present study we investigated the traffic of btGLUT4, a GLUT4-homolog identified in brown trout skeletal muscle (Planas et al., 2000a), and its regulation by insulin in muscle cells. In order to carry out a direct comparison between the traffic of btGLUT4 and that of its rat homolog we decided to use the same cell background to express both transporters. The rat muscle L6 cell line has been well characterized in terms of insulin response and glucose transport regulation (Bandyopadhyay et al., 1997; Beguinot et al., 1986; Ramlal et al., 1988; Walker et al., 1989). Generation of a stable L6 cell line expressing the myc-tagged rat GLUT4 has provided an useful tool to study

the intracellular traffic of GLUT4 and its regulation by insulin in mammalian skeletal muscle cells (Foster et al., 2001; Li et al., 2001; Randhawa et al., 2000; Randhawa et al., 2004; Rudich and Klip, 2003). Thus, we generated several stable cell lines of L6 cells that overexpress the myc-tagged btGLUT4 (L6-btGLUT4myc).

Tagging GLUT4 protein in an exofacial domain with the myc epitope allows measuring the amount of transporter inserted at the plasma membrane in intact cells by an easy and highly sensitive method (Wang et al., 1998), and also allows discerning the endogenous GLUT4 from the expressed GLUT4. Steady-state levels of btGLUT4myc and ratGLUT4myc, used as a control, at the cell surface were examined in response to insulin and a hyperosmotic shock. The stimulatory effects of insulin and hypertonicity enhancing ratGLUT4myc translocation are well known (Antonescu et al., 2005; Huang et al., 2005; Li et al., 2001; Randhawa et al., 2004; Somwar et al., 2001). Our results indicate that insulin stimulates the translocation of btGLUT4myc to the plasma membrane. This is not the first demonstration that insulin is able to cause the translocation of fish GLUT4. We have recently described the translocation of okGLUT4 (a GLUT4-homolog in salmon) to the plasma membrane in response to insulin when expressed in 3T3-L1 cells (Capilla et al., 2004). Therefore, fish GLUT4, like mammalian GLUT4, is regulated by insulin in terms of its translocation to the plasma membrane although with minor differences. Interestingly, the net gain of btGLUT4myc molecules at the cell surface caused by insulin in L6 myoblasts was lower than for ratGLUT4myc. This difference in the effects of insulin is probably due to the fact that under basal conditions there is a higher percentage of btGLUT4myc residing at the plasma membrane in btGLUT4myc-expressing L6 myoblasts, as shown by our results from translocation and internalization assays. A similar observation was reported by Capilla et al. (2004) in 3T3-L1 adipocytes transiently expressing okGLUT4, which also showed higher levels of transporter at the plasma membrane in the basal state than those that were expressing rat GLUT4. In contrast to previously reported (Niu et al., 2003), ratGLUT4myc translocation observed in myotubes in response to insulin was lower than in myoblasts and similar to that of btGLUT4myc.

Our results on the time-related effects of insulin on GLUT4myc translocation indicate that L6-btGLUT4myc cells need a longer exposure to the hormone than L6-ratGLUT4myc cells to show a significant translocation of GLUT4myc to the plasma membrane. After a 5-minute incubation with insulin, L6-ratGLUT4myc cells already showed a significant increase of ratGLUT4myc at the cell surface; whereas, L6-

btGLUT4myc cells required at least a 10-minute incubation with the hormone. This observation supports the hypothesis that these two transporters differ in their intracellular traffic since btGLUT4 has some differences in its amino acid sequence that could affect its interaction with regulatory proteins involved in the sorting and targeting of mammalian GLUT4 to the plasma membrane. On the other hand, the short time of insulin stimulation (10 minutes) required to cause btGLUT4 translocation is consistent with the rapid stimulation of glucose uptake observed in trout muscle cells after 15 minutes of incubation with insulin (Díaz and Planas, unpublished data; see chapter IV). Likewise, our results in the time course of insulin-stimulated ratGLUT4myc translocation agree with previous studies (Niu et al., 2003).

Hyperosmolarity effects on GLUT4myc translocation were also examined in myoblasts and myotubes. Intriguingly, btGLUT4myc seems to be sucrose-unresponsive in terms of translocation in the three clones studied, both on L6 myoblasts and myotubes. This lack of response of btGLUT4myc in hyperosmolarity-stimulating conditions is not due to an insufficient incubation time. Prolonged treatment with sucrose (40 minutes) did not produce an increase of surface btGLUT4myc, but btGLUT4myc levels at the cell surface even slightly diminished below basal levels. This drop of btGLUT4myc at the plasma membrane cannot be explained by cellular damage caused by the hyperosmotic shock, since this drop was not observed in L6 myoblasts that overexpress rat GLUT4. In contrast, hypertonicity increases ratGLUT4myc at the plasma membrane of myoblasts as it has been described previously (Li et al., 2001; Randhawa et al., 2004) in a similar manner than insulin but, surprisingly, hyperosmolarity does not have any effect on myotubes. In our experimental conditions we were unable to see a sucrose effect on ratGLUT4myc translocation in myotubes in any of the experiments performed. It is well known that a hyperosmotic shock increases the amount of mammalian GLUT4 at the plasma membrane preventing its endocytosis from the cell surface (Li et al., 2001) and, to a lesser degree, enhancing its externalization rate (Randhawa et al., 2004). Li et al. (2001) suggested that hyperosmolarity inhibits mammalian GLUT4 endocytosis presumably preventing the formation of clathrin-coated vesicles (Hansen et al., 1993; Heuser and Anderson, 1989). Therefore, the unresponsiveness of btGLUT4myc to hyperosmolarity suggests that btGLUT4, unlike rat GLUT4, could be internalized via a clathrin-independent pathway.

We also investigated insulin-stimulated glucose uptake in L6-btGLUT4myc cells compared to L6-ratGLUT4myc cells. Glucose uptake experiments were carried out in parallel with untransfected (wild-type) L6 cells to take into account the glucose uptake due to the endogenous expression of glucose transporters. All the btGLUT4myc-expressing clones in the myoblast and myotube stage show a similar increase on glucose uptake in response to insulin, and also similar to that of wild type and ratGLUT4myc cells. However, the absolute glucose transport rate differs considerably between the cell lines. All the clones that express btGLUT4myc had a lower glucose uptake than L6-ratGLUT4myc cells and it was directly correlated to the expression levels of btGLUT4myc protein in each clone. This feature is consistent with the lower level of GLUT4myc expression in btGLUT4myc clones compared to ratGLUT4myc cell line. Another explanation for these low values of glucose uptake in btGLUT4myc clones is that fish GLUTs have less affinity for glucose than mammalian GLUTs, at least GLUT4 and GLUT1 isoforms (Capilla et al., 2004; Teerijoki et al., 2001a). The low expression of the transporter and low affinity of btGLUT4 for glucose may explain that clones #7 and #9 have a basal glucose intake similar to the untransfected L6 cells. Nevertheless, in all the cell lines, including wild-type and L6-ratGLUT4myc, myotubes showed a lower glucose uptake rate than myoblasts according to previously reported (Niu et al., 2003; Ueyama et al., 1999). Interestingly, L6-btGLUT4myc cells showed an increase on glucose transport in response to hyperosmotic shock, but in a lesser extent than L6-ratGLUT4myc cells. This effect is probably due to the response of endogenous transporters to hyperosmolarity, an effect that we could not observe in translocation, because we were only detecting myc-tagged transporters. In clone 9 cells (an epithelial cell line from rat liver) hyperosmolarity induces GLUT1 translocation and also cause GLUT1 activation (Barros et al., 2001).

Glucose transport sensitivity to cytochalasin B was also compared between L6-ratGLUT4myc cells and L6 cells that overexpress btGLUT4myc. Cytochalasin B is a known inhibitor of facilitated glucose transport and, specifically, an endofacial inhibitor of GLUT4 (Ribe et al., 2005). In L6-btGLUT4myc-expressing myoblasts, cytochalasin B caused a half-maximal inhibition of glucose uptake at 1 μM, exactly as was described previously for *Xenopus* oocytes expressing okGLUT4 (Capilla et al., 2004). However, this same concentration of cytochalasin B caused an 87% inhibition of glucose uptake in L6-ratGLUT4myc myoblasts, indicating that cytochalasin B is less effective inhibiting glucose transport in btGLUT4myc-expressing myoblasts than in

ratGLUT4myc-expressing myoblasts. Considering that both cell lines share the same cell background these differences in cytochalasin B sensitivity can only be caused by the overexpressed GLUT4. Therefore these results suggest that btGLUT4 is less sensitive to cytochalasin B due to some differences in amino acid sequence and/or conformation of the cytochalasin B binding site.

We also examined GLUT4myc internalization in L6 myoblasts from the different cell lines. Our results indicate that the amount of btGLUT4myc remaining at the cell surface after 2 minutes of endocytosis is higher than that of ratGLUT4myc, at least for clones # 6 and # 7; clone # 9 also showed a higher amount of btGLUT4myc at the plasma membrane but the difference was not statistically significant. Furthermore, insulin appears to partially inhibit only ratGLUT4myc internalization. This observation does not agree with previous studies with L6-ratGLUT4myc myoblasts where insulin does not delay ratGLUT4myc internalization in the first 5 minutes (Foster et al., 2001; Li et al., 2001).

These differences between mammalian and fish GLUT4 in terms of traffic could be related to differences in the sequence of certain motifs shown to be important for internalization and intracellular retention of GLUT4. The dileucine motif at the carboxyl-cytoplasmic tail of mammalian GLUT4 has been described as crucial for its internalization from the plasma membrane as well as for its intracellular retention (Corvera et al., 1994; Haney et al., 1995; Verhey and Birnbaum, 1994; Verhey et al., 1995). Furthermore, it has been reported that a peptide comprising the dileucine signal in the C-terminus of GLUT4 interacts with the clathrin adapter complex AP-1 (Rapoport et al., 1998). Interestingly, these two leucines at positions 489 and 490 are missing in the btGLUT4 sequence. This feature could explain the slower endocytosis found in btGLUT4myc clones and also the higher percentage of btGLUT4myc at the cell surface of L6 myoblasts in the basal state. Moreover, the dileucine motif has been involved in GLUT4 targeting from TGN to the GLUT4 storage compartment (Martinez-Arca et al., 2000; Sandoval et al., 2000). Therefore, the absence of this motif in btGLUT4 protein could be the reason for the lower insulin response observed in btGLUT4myc cells in terms of translocation. Probably the lack of these two leucines may provoke some degree of mistargeting of btGLUT4 out of the specialized compartment that make it to be directed to the plasma membrane. Furthermore, btGLUT4 also lacks two arginine residues located at positions -4 and -5 upstream from the dileucine signal that have been implicated along with the dileucine motif in the

intracellular sorting and endocytosis of mammalian GLUT4 (Sandoval et al., 2000). On the other hand, some authors have pointed to the importance of the FQQI motif, located at the amino terminus, in the intracellular localization of GLUT4 (Al-Hasani et al., 2002; Garippa et al., 1994; Khan et al., 2004; Marsh et al., 1995; Piper et al., 1993). In the btGLUT4 sequence this motif is partially conserved (FQHL) but still contains the phenylalanine residue that it seems to be essential for a proper GLUT4 sorting.

Further investigation will be needed to elucidate the sequence signals that determine this different traffic of the brown trout GLUT4 with respect to that of rat GLUT4. It would be interesting to mutate btGLUT4 to restore the dileucine motif and see if its traffic resembles that of rat GLUT4. Likewise, unresponsiveness of btGLUT4 to a hyperosmotic shock in terms of translocation is an intriguing feature and its study could provide new clues for a better understanding of the traffic regulation of the mammalian GLUT4.

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CAPÍTOL IV: REGULACIÓ DE LA TRANSLOCACIÓ DEL GLUT4 I EL TRANSPORT DE GLUCOSA PER LA INSULINA EN CÈL·LULES MUSCULARS DE TRUITA

REGULACIÓ DE LA TRANSLOCACIÓ DEL GLUT4 I EL TRANSPORT DE GLUCOSA PER LA INSULINA EN CÈL·LULES MUSCULARS DE TRUITA

Resum

El GLUT4 és el principal transportador de glucosa responsable de l'augment d'activitat transportadora de glucosa que té lloc amb l'estimulació de la insulina en les cèl·lules musculars de mamífers. En resposta a la insulina el GLUT4 de mamífer és reclutat cap a la membrana plasmàtica on exerceix la seva funció. En peixos s'ha identificat un transportador de glucosa homòleg al GLUT4 de mamífer i s'ha demostrat que aquest transportador és capaç de ser translocat cap a la superfície cel·lular en resposta a la insulina quan és expressat en cèl·lules de mamífer. A més, es coneix que la insulina estimula la captació de glucosa en cèl·lules musculars de truita però encara es desconeix si aquest increment de l'activitat transportadora de glucosa és la conseqüència de l'increment en el nombre de molècules de GLUT4 a la membrana plasmàtica. Per tant, l'objectiu d'aquest treball va ser analitzar l'efecte de la insulina sobre la translocació del GLUT4 de peix i determinar la seva distribució subcel·lular en cèl·lules musculars de truita.

FEBS Letters (in preparation)

REGULATION OF GLUT4 TRANSLOCATION AND GLUCOSE TRANSPORT BY INSULIN IN TROUT MUSCLE CELLS

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Running title: GLUT4 translocation in trout muscle cells

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ABSTRACT

GLUT4 is the main glucose transporter responsible for the increase on glucose transport activity upon insulin stimulation in skeletal muscle cells of mammals. In response to insulin mammalian GLUT4 is recruited to the plasma membrane where exerts its function. In fish, a glucose transporter homologous to mammalian GLUT4 has been identified in fish and it has been demonstrated that this transporter is able to translocate to the cell surface in response to insulin when expressed in mammalian cells. Furthermore, it is known that insulin stimulates glucose uptake in trout muscle cells but it remains unknown whether or not this enhanced glucose transport activity is a consequence of an increase in the number of GLUT4 molecules at the plasma membrane. Thus, the aim of this study was to analyze the effect of insulin on fish GLUT4 translocation and to determine the subcellular distribution of fish GLUT4 in trout muscle cells.

INTRODUCTION

In mammals, GLUT4 is the insulin-regulated glucose transporter and is mainly expressed in tissues, such as skeletal muscle and adipose tissue, that are able to respond to insulin. It is widely known that insulin increases glucose uptake in its target tissues in order to maintain glucose homeostasis. At the early eighties, before the cloning and identification of GLUT4, some authors already demonstrated that insulin induced the translocation of glucose transporters to the plasma membrane in muscle and fat cells (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Wardzala and Jeanrenaud, 1981). Some years after these initial findings, several studies demonstrated that insulin stimulates glucose uptake in adipose and muscle cells increasing the amount of GLUT4 at the plasma membrane (James et al., 1989; Mitsumoto and Klip, 1992). Thus, in absence of stimulus, GLUT4 molecules have a cytoplasmic localization, mainly concentrated at the perinuclear region, and insulin causes a redistribution of the transporter promoting its exposure at the cell surface (Thong et al., 2005; Watson et al., 2004a). The regulation of GLUT4 translocation by insulin is a complex mechanism in which a high number of signaling molecules are involved and is a research area still under intense investigation. Several pieces of evidence suggest that the PI 3-kinase/Akt pathway participates in the recruitment of GLUT4 to the plasma membrane upon insulin stimulation (Chang et al., 2004). Besides promoting GLUT4 translocation, it has been suggested that insulin also enhances GLUT4 intrinsic activity (Furtado et al., 2003). This hypothesis arose from the discrepancy between the magnitude of GLUT4 translocation and glucose transport in muscle cells (Furtado et al., 2003), and also from the temporal segregation of both phenomena (Somwar et al., 2001). Thus, in mammals, insulin would increase glucose uptake in its target tissues through the recruitment of GLUT4 molecules to the cell surface and their subsequent activation.

In fish, glucose transporters homologous to mammalian GLUT4 have also been identified. In particular, our group has reported on the cloning of a GLUT4-homolog in skeletal muscle from brown trout (btGLUT4) (Planas et al., 2000a) and in adipose tissue from salmon (okGLUT4) (Capilla et al., 2004). The salmonid GLUT4 molecules identified share a high degree of sequence similarity with mammalian GLUT4 sequences (around 80%) and have been shown to be regulated by insulin. Recently, we have demonstrated that btGLUT4 gene expression is regulated *in vivo* by circulating

insulin levels in red skeletal muscle (Capilla et al., 2002). Furthermore, we have demonstrated that insulin stimulates GLUT4 expression by acting directly on muscle cells (Díaz and Planas, unpublished observations; see chapter II). In addition, recent studies in trout muscle cells and adipocytes have demonstrated that insulin causes an increase on glucose uptake rate in these cells (Capilla et al., 2004; Castillo et al., 2004). Since skeletal muscle is the main site for glucose uptake in fish (Blasco et al., 1996) we were interested to study the possible short-term effects of insulin on GLUT4 translocation in trout muscle cells in culture. Previous studies from our laboratory have shown up that fish GLUT4 can be stimulated by insulin to translocate to the plasma membrane in mammalian cells. In particular, insulin has been shown to induce okGLUT4 translocation to the plasma membrane when is transiently expressed in 3T3-L1 adipocytes (Capilla et al., 2004), and also to induce the translocation to the plasma membrane of btGLUT4 stably expressed in L6 muscle cells (Díaz and Planas, unpublished data; see chapter III). These studies have been performed expressing fish GLUT4 in a heterologous system such as mammalian cell lines. However, it remains unclear whether or not fish GLUT4 is able to translocate to the plasma membrane upon insulin stimulation in fish cells. Thus, the aim of this work was to determine if btGLUT4 can be recruited to the plasma membrane in response to insulin in trout muscle cells in culture leading to an increase on glucose transport activity of these cells.

MATERIAL AND METHODS

Materials

Media and chemicals for cell culture were obtained from Sigma (Tres Cantos, Spain). Cell culture plates were from Nunc (Roskilde, Denmark). Salmon insulin was kindly supplied by Dr. E. M. Plisetskaya (University of Washington, Seattle, United States). Cationic silica was a kind gift of Dr. David E. James (Garvan Institute of Medical Research, Sydney, Australia). Nycodenz was from Sigma. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from Jackson Immunoresearch (Soham, United Kingdom). AlexaFluor488-conjugated goat anti-rabbit IgG was obtained from Invitrogen (Prat del Llobregat, Spain). 2-Deoxy-D-[2,6-^{3}H]glucose was purchased from Amersham Biosciences (Barcelona, Spain). Immunofluore mounting

medium was from ICN (Madrid, Spain). Indinavir was kindly provided by Merck (Haarlem, The Netherlands).

Animals

Brown trout of 5-10 g were obtained from the Piscifactoria de Bagà (Barcelona, 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^{o}C. Animals were fed *ad libitum* with a commercial diet and fasted 24 h prior to the experiments.

Isolation of muscle satellite cells from brown trout (*Salmo trutta*)

Animals (40 to 80 for each culture) were sacrificed by a blow to the head and immersed in 70% ethanol for 30 seconds in order to sterilize external surfaces. Muscle satellite cells were isolated and cultivated following a protocol described previously (Castillo et al., 2002; Fauconneau and Paboeuf, 2000) with slight modifications. After removal of the skin, dorsal white muscle was isolated in sterile conditions and collected in DMEM medium containing 9 mM $NaHCO_3$, 20 mM Hepes, 15% horse serum and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B, 75 μg/ml gentamycin). After mechanical dissociation of the muscle in small pieces, the tissue was enzymatically digested with a 0.2% collagenase solution in DMEM for 1 h at 18^{o}C with gentle shaking. The suspension was centrifuged (300 x g for 10 min at 15^{o}C) and the resulting pellet was subjected to two rounds of enzymatic digestion with a 0.1% trypsin solution in DMEM for 20 min at 18^{o}C and gentle agitation. After each round of trypsinization the suspension was centrifuged and the supernatant was diluted in 4 volumes of cold DMEM supplemented with 15% horse serum and the same antibiotic-antimycotic cocktail mentioned before. After two washes with DMEM, the cellular suspension was filtered through 100 and 40 μm nylon filters. Cells were counted and diluted with DMEM containing 10% FBS to reach a final concentration of $4x10^6$ cells/ml.

Cell culture

Plates and coverslips were pretreated with 100 μg/ml poly-L-lysine (MW 70000-150000, Sigma) at a concentration of 4 μg/cm^2 for 5 min at 18^{o}C. The excess of

poly-L-lysine was removed and plates were washed with distilled water and air-dried for 1 h. After this period, plates and coverslips were incubated with 20 μg/ml laminin for 24 h at 18°C. Laminin solution was aspirated just before plating the cell suspension. Cells were cultured on 6-well plates for plasma membrane preparation or 12-well plates for 2-deoxyglucose assay at a density of 3-4x10^6 and 1x10^6 cells/well, respectively.

Cells were maintained at 18°C with DMEM containing 9 mM $NaHCO_3$, 20 mM Hepes, 10% fetal bovine serum and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B). After 24 h of plating, plates were washed to eliminate those cells not adhered to the well. Medium was routinely renewed each 48 h. All cultures were morphologically monitored by observation with an inverted microscope in order to control the state of the cells.

Plasma membrane isolation

Plasma membrane preparations were obtained as described in Chaney and Jacobson (1983) and Larance et al. (2005). Trout muscle cells at day 5 of culture were serum-deprived for 4 h and incubated in the absence or presence of 1 μM salmon insulin at 18°C for 30 min. After washing the cells with ice-cold PBS, cells were rinsed twice with ice-cold coating buffer (20 mM MES, 150 mM NaCl, 280 mM sorbitol, pH 5.0-5.5) and cationic silica 1% in coating buffer was added to the cells for 2 min at 4°C. The excess of silica was removed and cells were washed with ice-cold coating buffer. Following an incubation with 1 mg/ml polyacrylic acid for 2 min at 4°C, cells were washed with coating buffer and lysed with modified HES buffer (20 mM HEPES, 250 mM sucrose, 1 mM DTT, 1 mM magnesium acetate, 100 mM potassium acetate, 0.5 mM zinc chloride, pH 7.4). The lysate was passed 12 times through a 22-gauge needle and 6 times through a 27-gauge needle. One volume of nycodenz (100% in modified HES buffer) was added to the lysate that was layered onto 0.5 ml 70% nycodenz and centrifuged in a swing-out rotor at 41545 x g for 20 min at 4°C. The pellet was resuspended in modified HES buffer and spun at 500 x g for 5 min at 4°C three times to remove all nycodenz. The final pellet was resuspended in Laemmli sample buffer, heated at 65°C for 10 min and centrifuged at 10000 x g for 5 min. The resulting supernatant was stored at -80°C until immunoblot analysis.

Electrophoresis and immunoblotting

Plasma membrane samples (4 μg protein) were subjected to 12% SDS-PAGE and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked with blocking buffer (Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk) for 2 h and subsequently incubated overnight with a polyclonal anti-okGLUT4 antibody (Capilla et al., 2004) diluted to 1:500 in blocking buffer at 4^{o}C. After several washes the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by chemiluminiscence and quantified with an image analyzer (TotalLab v.1.11).

2-Deoxyglucose uptake assay

After 5 days in culture, trout muscle cells were serum starved for 4 h and subsequently incubated in the absence or presence of salmon insulin (100 or 1000 nM) for 5, 15 and 30 min at 18^{o}C. Cells were washed twice with PBS and were incubated with HEPES buffered saline containing 50 μM 2-deoxyglucose (2 μCi/ml 2-[^{3}H]-deoxyglucose) for 30 min at 18^{o}C. After this period, transport solution was removed and cells were rinsed three times with ice-cold PBS containing 50 mM glucose. Finally, cells were lysed with 0.1 N NaOH, 0.1% SDS and radioactivity was determined by scintillation counting. Protein concentration was measured by the Bradford method (Bradford, 1976). Non-specific uptake was carried out with the presence of 10 μM cytochalasin B in the transport solution, and these values were subtracted of all other values. In the experiments with indinavir, this inhibitor was added to the transport solution in a final concentration of 100 μM.

Immunofluorescence

To perform immunofluorescence analysis, brown trout muscle satellite cells were grown on glass coverslips previously coated with poly-L-lysine and laminin. Cells were rinsed with PBS and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. After washing three times, cells were sequentially incubated with 100 mM glycine in PBS for 10 min and permeabilized with 0.1% saponin in PBS for 20 min. All the subsequent incubations and washes were made in the presence of 0.1% saponin. Cells were blocked with 10% goat serum for 20 min and incubated with the primary

antibody against fish GLUT4 diluted 1:50 in blocking solution for 1 h at room temperature. After several washes with 0.1% saponin in PBS cells were incubated with 4 μg/ml AlexaFluor488-conjugated secondary antibody for 1 h at room temperature. Coverslips were washed and mounted with Immunofluore mounting medium. Confocal images were obtained with a Leica SPII laser confocal fluorescence microscope with a 63x objective.

Statistical analysis

Results are given as means ± SE. Data were analyzed using Statview 5.0. Differences between groups were evaluated by Student's t-test.

RESULTS

btGLUT4 translocation in response to insulin in trout muscle cells

In order to examine the possible effect of insulin on the subcellular redistribution of btGLUT4 we analyzed by western blot the amount of GLUT4 present in purified plasma membrane fractions from trout muscle cells previously incubated in the absence or presence of 1 μM insulin for 30 minutes. Our results indicate that btGLUT4 content at the plasma membrane was significantly stimulated by insulin in trout muscle cells (Fig. 1).

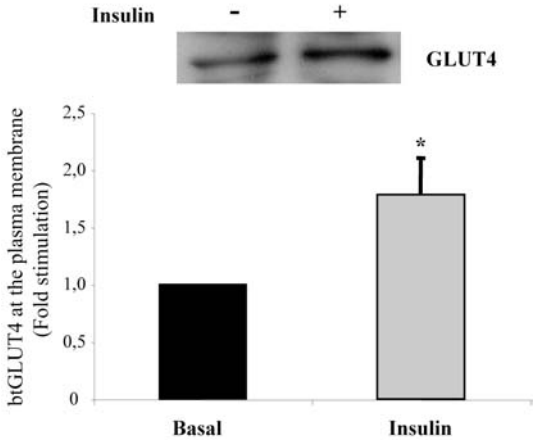

Fig. 1. Effect of insulin on the amount of btGLUT4 at the plasma membrane. After 5 days of culture trout muscle cells were serum-starved for 4 h and subsequently incubated in the absence or presence of 1 μM insulin for 30 min at 18°C. After the incubation period plasma membranes were obtained as described under "Materials and methods". *Top*, a representative immunoblot is shown. *Bottom*, densitometric analysis of four independent experiments. Values are mean ± SE and were set to 1 in the basal group. *Significant differences compared to basal (P<0.05).

2-deoxyglucose uptake in response to insulin in trout muscle cells

Glucose uptake measurements in trout muscle cells were conducted to determine the functional consequences of the increased amount of btGLUT4 at the cell surface upon insulin stimulation. A time course experiment of insulin-stimulated glucose uptake was performed on day-5 trout myocytes and two concentrations of insulin were assessed (100 and 1000 nM) (Fig. 2A).

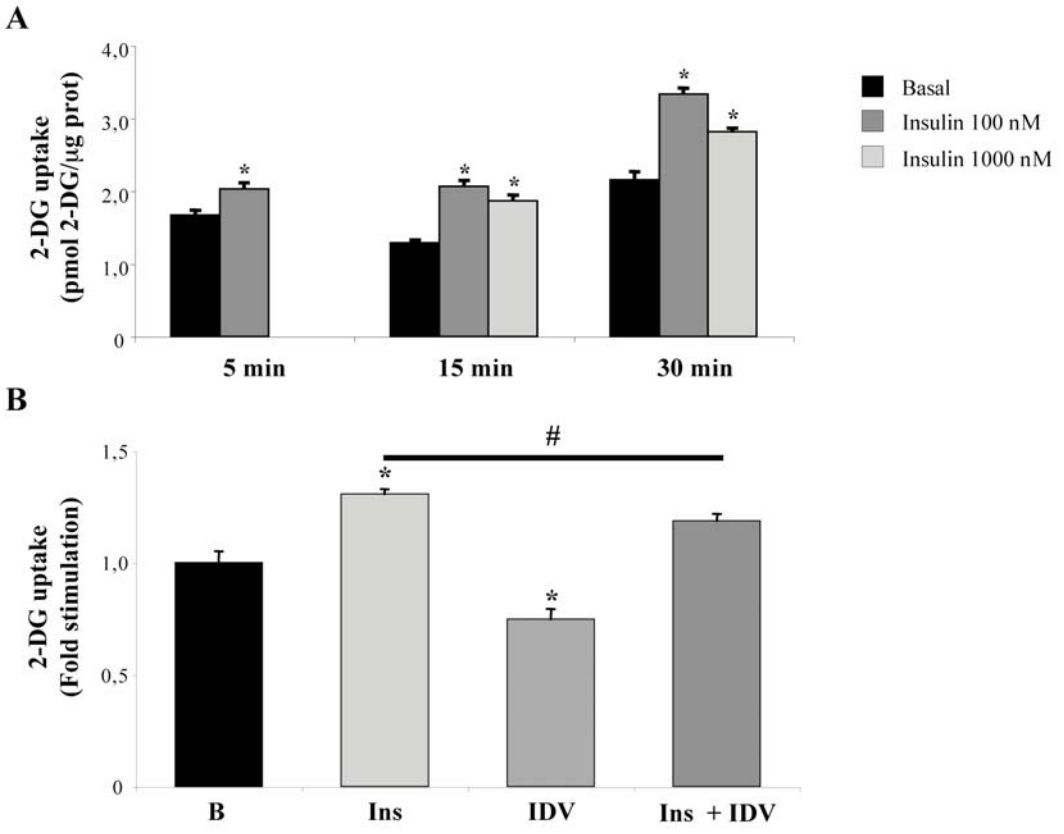

Fig. 2. *A*, Time course of insulin-stimulated 2-deoxyglucose uptake in trout muscle cells. After 5 days of culture cells were serum-starved for 4 h and subsequently incubated for the indicated times with or without insulin (100 or 1000 nM) at 18°C. After the incubation period 2-deoxyglucose uptake assay was performed as indicated under "Material and methods". Results are expressed as absolute values of pmoles of 2-DG per μg of protein. *B*, Effect of indinavir on insulin-stimulated 2-deoxyglucose uptake. After 5 days of culture cells were serum-starved for 4 h and subsequently incubated in the absence or presence of 1 μM insulin for 30 min at 18°C. After the incubation period 2-deoxyglucose uptake assay was performed as indicated under "Material and methods". Indinavir was added to the transport solution in a final concentration of 100 μM. Results are expressed as fold stimulation above basal which was set to 1. Values are mean ± SE from a representative experiment performed in triplicate. * Significant differences compared to basal (P<0.05). # P<0.05.

In trout muscle cells, the stimulatory effects of insulin on 2-deoxyglucose uptake were already visible after 5 minutes of insulin (100 nM) stimulation and increased with the incubation time. The highest concentration of insulin (1000 nM) had a similar effect than 100 nM insulin at 15 minutes of incubation and it was less effective than 100 nM at 30 minutes.

We also assessed the effects of indinavir (a mammalian GLUT4 inhibitor) in basal and insulin-stimulated 2-deoxyglucose uptake in primary cultures of trout muscle cells (Fig. 2B). The presence of indinavir during the glucose uptake assay caused a 25% reduction in basal glucose uptake rate. Indinavir also decreased the insulin-stimulated glucose uptake, but only a 9% reduction was observed.

Immunofluorescence detection of btGLUT4 in trout muscle cells

Primary cultures of brown trout muscle cells were examined by immunofluorescence to determine the subcellular localization of btGLUT4 in the basal state. As shown in Fig. 3A, btGLUT4 immunoreactivity was clearly localized in intracellular compartments, showing a preferential distribution in the perinuclear region. In some cells, btGLUT4 immunoreactivity was also detected in the plasma membrane.

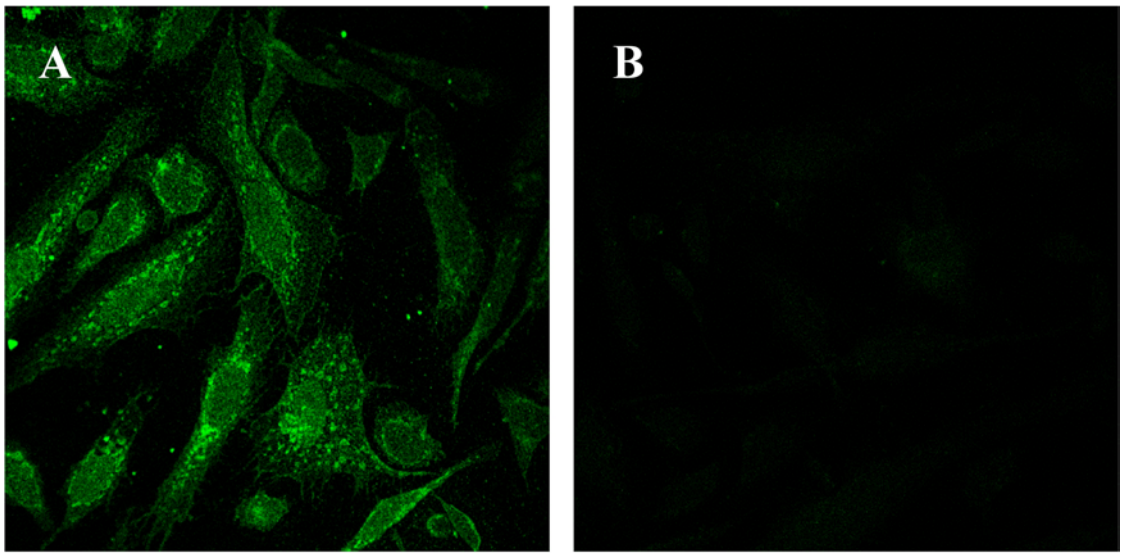

Fig. 3. Subcellular localization of btGLUT4 in trout muscle cells. *A*, Immunofluorescence of btGLUT4 in cells at day 5 of culture using the anti-okGLUT4 as primary antibody and AlexaFluor488-conjugated antibody. *B*, Background fluorescence (in the absence of primary antibody).

Fig. 3B shows the staining in absence of primary antibody, indicating that the staining in Fig. 3A was not due to a nonspecific signal from the secondary antibody. We also compared the immunofluorescence detection of btGLUT4 in the basal state between 5-day-cultured myocytes and 10-day-cultured myotubes. In both differentiation stages, the localization of btGLUT4 was mainly intracellular (Fig. 4A, C). Furthermore, the labeling for btGLUT4 was more intense in fully differentiated myotubes than in muscle cells at day 5 of culture.

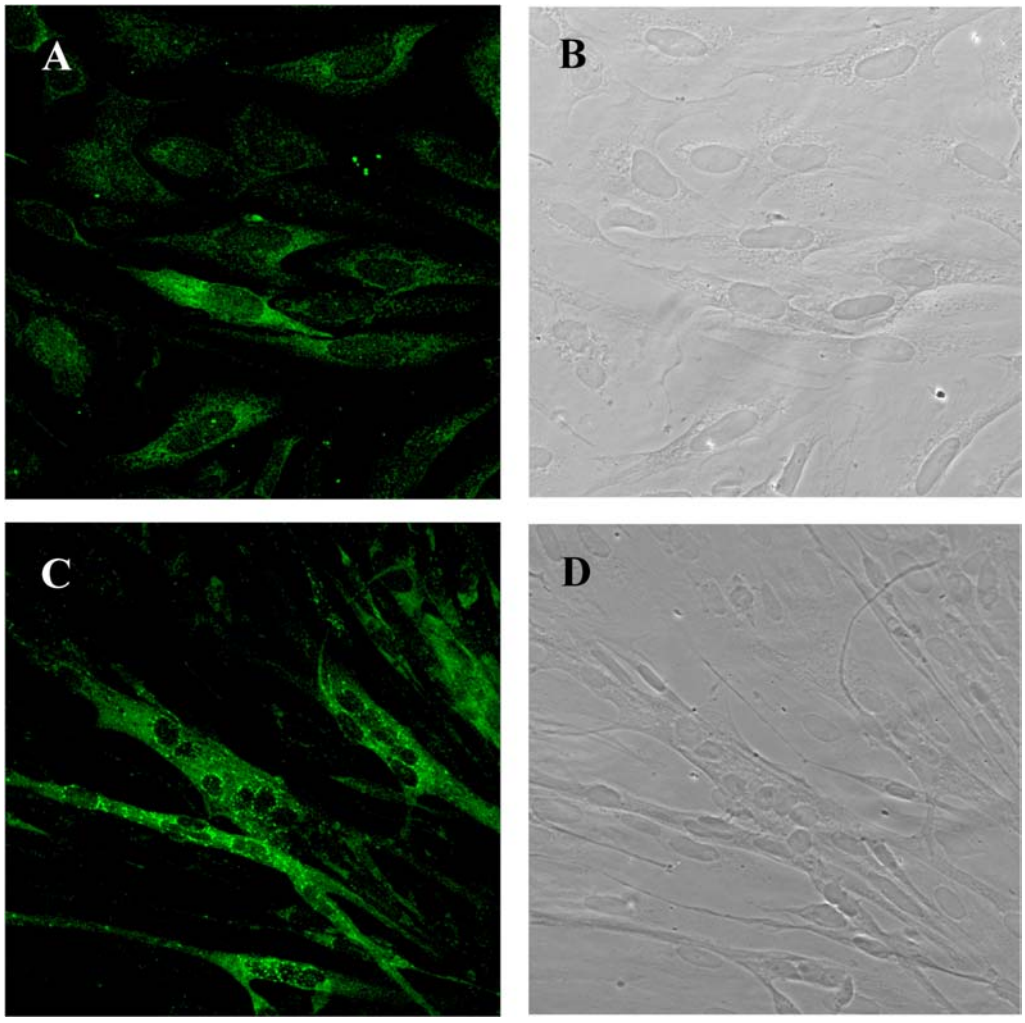

Fig. 4. Immunofluorescence of btGLUT4 in trout muscle cells after 5 days (A-B) or 10 days (C-D) in culture. In the figure are shown the immunofluorescence images (A, C) along with the corresponding phase-contrast images (B, D).

DISCUSSION

In this study we investigated the role of insulin in the subcellular localization of trout GLUT4 and in the glucose uptake rate in primary cultures of trout muscle satellite cells. Our findings clearly indicate that insulin stimulates the appearance of btGLUT4 at the plasma membrane and, as consequence, enhances glucose uptake in trout muscle cells. Therefore, muscle cells incubated with insulin exhibited a higher amount of btGLUT4 at the cell surface than non-stimulated cells, most likely as a result of the stimulatory effect of insulin on GLUT4 trafficking to the plasma membrane. Previous studies in our laboratory have described the translocation of fish GLUT4 to the plasma membrane in response to insulin when is expressed in mammalian cells (3T3-L1 adipocytes and L6 muscle cells) (Capilla et al., 2004; Díaz and Planas, unpublished observations, see chapter III). However, this is the first study reporting on the stimulatory effects of insulin on btGLUT4 translocation in fish muscle cells. In mammals, a large number of studies have described the GLUT4 translocation stimulated by insulin in muscle and adipose cells (Al-Khalili et al., 2003; Guillet-Deniau et al., 1994; James et al., 1989; Mitsumoto and Klip, 1992; Suarez et al., 2001). Therefore, it seems that GLUT4 translocation is a well-conserved effect of insulin in skeletal muscle cells among vertebrates.

The increase of btGLUT4 at the plasma membrane of trout muscle cells in response to insulin should be followed by an increase in their glucose uptake rate. We performed 2-deoxyglucose uptake assays in trout muscle cells to determine the effects of insulin on the ability of these cells to take up glucose. Trout muscle cells in culture were incubated in the absence or presence of insulin for 5, 15 or 30 minutes followed by glucose transport assay for 30 minutes. Our results indicate that insulin promotes glucose uptake in muscle cells from brown trout, in agreement with previously reported studies using primary cultures of muscle satellite cells from rainbow trout (Castillo et al., 2004). Likewise, insulin-stimulated glucose uptake has also been observed in muscle satellite cells from chicken (Duclos et al., 1993), sheep (Roe et al., 1995) and human (Al-Khalili et al., 2003; Sarabia et al., 1992) as well as in muscle cell lines (del Aguila et al., 1999; Mitsumoto et al., 1991; Palmer et al., 1997). The time course of insulin-stimulated glucose uptake in trout muscle cells suggests that the effect of insulin increasing glucose transport is rapid, since a significant increase is already detected after 5 minutes of insulin stimulation. In the same way, other studies have also reported

an enhanced glucose uptake rate by short-time incubations with insulin in L6 muscle cells (Niu et al., 2003; Somwar et al., 2001). Furthermore, the rapid stimulation of glucose uptake by insulin detected in trout muscle cells correlates with the rapid translocation of btGLUT4 to the plasma membrane observed in L6 cells stably expressing btGLUT4 (Díaz and Planas, unpublished data; see chapter III). Although we only checked btGLUT4 translocation after 30 minutes of insulin stimulation, the existence of insulin-stimulated glucose uptake with shorter incubations suggests that trout GLUT4 could be recruited to the cell surface with shorter insulin stimulation periods.

In order to determine if the insulin-stimulated glucose uptake is, at least in part, mediated by GLUT4, we used indinavir, which is an HIV protease inhibitor that has been described as a selective inhibitor for the GLUT4 isoform in mammals (Murata et al., 2002). This drug preferentially inhibits GLUT4-mediated glucose transport in a non-competitive manner when is added to the transport solution (Murata et al., 2002), but does not prevent GLUT4 translocation to the plasma membrane in L6 cells (Rudich et al., 2003). In brown trout muscle cells indinavir caused a significant reduction in both basal and insulin-stimulated glucose uptake in trout muscle cells, suggesting that GLUT4 is indeed involved, at least in part, in mediating the stimulatory effects of insulin on glucose uptake. The exact mechanism used by indinavir to inhibit mammalian GLUT4 is not clearly understood yet, but it has been suggested that indinavir binds directly and non-covalently to GLUT4, presumably on the cytosolic surface of the transporter (Hertel et al., 2004). In trout muscle cells indinavir caused a 25% and 9% reduction in basal and insulin-stimulated glucose uptake, respectively, whereas in L6 myotubes the same concentration of the inhibitor caused about 35% and 50% reduction of both basal and insulin-stimulated glucose transport activity (Rudich et al., 2003). Therefore, it appears that indinavir is less effective inhibiting trout GLUT4 than mammalian GLUT4. The difference in sensitivity could be due to the existing differences between the primary structure of trout and mammalian GLUT4. Likewise, different sensitivity to a known inhibitor of mammalian GLUTs as cytochalasin B has also been described for btGLUT4 (Díaz and Planas, unpublished data; see chapter III). Furthermore, GLUT4 is not the only GLUT isoform present in trout muscle cells, since these cells also express GLUT1 (Capilla et al., 2002; Díaz and Planas, unpublished results, chapter II). Since we do not know the relative sensitivity of trout GLUT1 to indinavir, it is difficult to conclude from these results what is the relative contribution of

trout GLUT4 to glucose transport activity in trout muscle cells. Further investigation will be needed to answer this question.

We also examined the subcellular localization of btGLUT4 on myocytes and fully differentiated myotubes by immunofluorescence. In the basal state, btGLUT4 distribution was mainly intracellular, located in punctate structures throughout the cytoplasm and also concentrated in the perinuclear region. Therefore, it seems that trout GLUT4 has a similar intracellular distribution than mammalian GLUT4 (Baque et al., 1998; Thong et al., 2005; Zeigerer et al., 2004). However, some muscle cells also showed the immunoreactivity of btGLUT4 at the plasma membrane. This observation agrees with previous studies in which fish GLUT4 is expressed in mammalian cells. In particular, Capilla et al. (2004) reported that 3T3-L1 adipocytes showed a higher percentage of okGLUT4 at the cell surface in the basal state than those that were expressing rat GLUT4. Similarly, we also detected that L6 cells show a higher percentage of btGLUT4 at the plasma membrane under basal conditions than those that were expressing rat GLUT4 (Díaz and Planas, unpublished data, see chapter III). Therefore, this btGLUT4 immunoreactivity at the plasma membrane of trout muscle cells could be due to the fact that there is a higher number of transporters at the cell surface in the basal state. In addition, btGLUT4 labeling was clearly more intense in myotubes than in myoblasts, according to the higher GLUT4 expression observed in trout muscle differentiated cells (Díaz and Planas, unpublished data; see chapter II).

In summary, this study provides the first evidence that brown trout GLUT4 is able to translocate to the plasma membrane in response to insulin in its endogenous environment, which is the trout muscle cell. Our results indicate that the action of insulin on the redistribution of glucose transporters has been conserved throughout the evolution of vertebrates. However, GLUT4 translocation is a complex mechanism in which a high number of factors are involved, and it is still under investigation. To date, the insulin signaling pathway that leads to the redistribution of trout GLUT4 from intracellular stores to the plasma membrane remains unknown but it is likely that it does not differ much from that reported in mammals. Recently, Castillo et al. (2006) demonstrated the activation of Akt by insulin in trout muscle cells, showing that this pathway is conserved among vertebrates. In addition, Capilla et al. (2004) reported the inhibition of insulin-stimulated glucose uptake by wortmannin (a PI 3-kinase inhibitor) in trout adipocytes, raising the possibility that these molecules participate in fish GLUT4 translocation induced by insulin. In conclusion, insulin stimulates the

appearance of fish GLUT4 at the plasma membrane of trout muscle cells and, therefore, increases the glucose uptake rate, likely by a signaling pathway similar to that occurs in mammalian cells.

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