Direct involvement of tumor necrosis factor α in the regulation of glucose uptake in rainbow trout muscle cells

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Short title: Metabolic effects of recombinant trout TNFα in trout muscle cells
Abstract

The proinflammatory cytokine tumor necrosis factor alpha (TNFα) is known to have a direct action on skeletal muscle in mammals. However, little is known regarding the potential effects of cytokines on non-immune tissues, particularly in skeletal muscle, in fish. The aim of this study was to investigate the effects of recombinant trout TNFα (rtTNFα) on skeletal muscle carbohydrate metabolism in rainbow trout (Oncorhynchus mykiss). We used a primary cell culture of muscle cells from rainbow trout to show that rtTNFα stimulates glucose uptake in myoblasts and myotubes at concentrations that do not affect the viability of the cells, requiring de novo protein synthesis as shown by the impairment of rtTNFα-stimulated glucose uptake by cycloheximide. With the use of specific inhibitors we show that rtTNFα-stimulated glucose uptake is mediated by the p38MAPK, NF-κB and JNK pathways. Additionally, we provide evidence that the stimulatory effects of rtTNFα on glucose uptake in trout skeletal muscle cells may be caused, at least in part, by an increase in the amount of GLUT4 at the plasma membrane. Incubation of trout muscle cells with conditioned medium from LPS-stimulated trout macrophages, enriched in TNFα, increased glucose uptake. Our results indicate that recombinant as well as native trout TNFα directly stimulates glucose uptake in trout muscle cells and provide evidence, for the first time in non mammalian vertebrates, for a potential regulatory role of TNFα in skeletal muscle metabolism.

Keywords: GLUT4, insulin, LPS, macrophage conditioned-media, cytokine
Introduction

Glucose entry and utilization by the vertebrate skeletal muscle, a major contributor to whole body glucose disposal, is stimulated by metabolic hormones among which insulin has a prevalent role. In mammals, the mechanisms by which insulin stimulates glucose entry into skeletal muscle, namely by increasing the plasma membrane localization and abundance of the insulin-regulatable glucose transporter GLUT4, are fairly well characterized (29). Recent studies in teleosts have provided evidence for evolutionary conserved insulin-regulated mechanisms of glucose entry into skeletal muscle. Specifically, glucose uptake in teleost (trout) skeletal muscle is stimulated by insulin in vivo and in vitro by increasing the abundance of GLUT4 at the plasma membrane and the expression of GLUT4 at the transcript and protein levels (10, 19-21). Interestingly, teleost GLUT4 has lower affinity for glucose and less efficient intracellular retention than mammalian GLUT4 (9, 11, 19) which could explain, at least in part, the so-called glucose intolerance attributed to the lower ability of teleosts to clear a glucose load (35).

In mammals, mediators other than classical metabolic hormones, such as cytokines, are also known to affect glucose entry into the skeletal muscle. Among these, the proinflammatory cytokine tumor necrosis factor alpha (TNFα) is one of the most studied for its catabolic effects in target cells during excessive production (i.e. sepsis) and for its proposed role in skeletal muscle mass loss in cancer and ageing (17, 32). TNFα is known to have a direct action on skeletal muscle and is reported to impair myogenic differentiation by suppressing MyoD and IGF-I-induced myogenin expression (8, 27). However, other reports indicate that TNFα can stimulate muscle cell proliferation, myoblast myogenic differentiation and protein synthesis (2, 13, 30) in line with the proposed role of this cytokine in muscle regeneration (17). With regards to
glucose homeostasis, high TNFα circulating levels are associated with insulin resistance and TNFα has been reported to inhibit insulin-stimulated glucose uptake in skeletal muscle cells in culture (16, 18). However, as with other actions of TNFα, other studies have shown opposite effects on glucose entry into muscle cells; namely, that TNFα stimulates glucose uptake (14, 40, 48) by increasing the cell surface levels of GLUT4 (40). It is increasingly clear that the cellular response to TNFα strongly depends on the concentration of TNFα used, on the time of exposure and the cellular background.

Despite a fair degree of divergence from mammalian TNFα in terms of its amino acid sequence, teleost fish also express and release a TNFα ortholog (34, 39). Over the last few years, recombinant TNFα proteins from several fish species have been produced and their effects on immune cells and tissues have been investigated (23, 24, 26, 28, 36, 38, 39, 45, 47, 49). However, there is no information available to date on the metabolic effects of homologous recombinant TNFα in non-immune cells or tissues from fish. The only available data on the potential metabolic effects of this cytokine is derived from studies showing stimulation of lipolysis from rainbow trout and seabream adipocytes in response to recombinant human TNFα (1, 41). Given that skeletal muscle, which accounts for more than 50% of the body weight, is the most important tissue for regulated glucose uptake in fish (5) and since it represents the edible portion of aquacultured fish destined for human consumption, it is important to investigate the mechanisms regulating the entry of glucose in this tissue. Therefore, the aim of the present study was to investigate the metabolic effects of recombinant trout TNFα in trout muscle cells in culture in relation to its potential effects on glucose uptake. Our results clearly indicate that this cytokine stimulates glucose uptake in trout myoblasts and myotubes and support the idea that TNFα is an important physiological factor
contributing to glucose homeostasis in vertebrates.

**Materials and Methods**

*Animals*

Rainbow trout (*Oncorhynchus mykiss*) of 8-14 g body weight were purchased from the Piscifactoria Truites del Segre (Oliana, Lleida, Spain). Animals 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 14°C. They were fed ad libitum with a commercial diet and fasted 24 h prior to the experiments. The experimental protocols used for trout in this study have been reviewed and approved by the Ethics and Animal Welfare Committee of the University of Barcelona, Spain.

*Isolation of trout muscle satellite cells*

Animals (40 to 80 for each isolation) were sacrificed by a blow to the head and skin was sterilised by immersion in 70% ethanol for 30 seconds. For the cell isolation, we followed the protocol described by Fauconneau and Paboeuf (22), with some modifications (19, 21). Briefly, the skin was removed and dorsal white muscle was isolated in sterile conditions and collected in DMEM medium at pH 7.4, containing 9 mM NaHCO$_3$, 20 mM Hepes, 15% horse serum, antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B) and 0.15% gentamycin. The tissue was mechanically dissociated and then enzymatically digested with a 0.2% collagenase solution in DMEM for 1 h at 18°C and gentle shaking. After centrifugation (300 g for 10 min at 15°C), the resulting pellet was washed in DMEM without horse serum and was enzymatically digested twice with a 0.1% trypsin solution in DMEM for 20 min at 18°C under gentle shaking. The resulting cellular suspension
was filtered through 100 and 40 μm nylon filters. The obtained cells were counted and plated on 12-well plates (BD Biosciences, Madrid, Spain) at a density of 1.8-2x10^6 cells/well. Plates were treated the day before with poly-L-lysine and laminin to facilitate cell adhesion. Media, enzymes and reagents used during the isolation were purchased from Sigma-Aldrich (Tres Cantos, Spain).

**Trout muscle satellite cell culture**

Trout muscle cells were maintained at 18°C under a humidified air atmosphere in DMEM containing 9 mM NaHCO₃, 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 non adherent cells. Medium was routinely renewed every second day, while cultures were monitored by daily observation under an inverted microscope (Zeiss Axiovert 25). Trout muscle cells were cultured for 2 and 10 days and at the day of the experiment cells were serum-starved for 4 h. Cells were incubated in the absence or presence of recombinant trout TNFα (rtTNFα), generated by expressing the mature form of rainbow trout TNFα in *E. coli* and shown to be biologically active in a fish cell line (39) and in trout ovarian tissue culture (15). Recombinant human insulin (Sigma) was added to the cells at a concentration of 1 μM for 60 min prior to the glucose uptake assays. SB220025, SP600125 and BAY 11-7082 inhibitors (Calbiochem, La Jolla, CA), used at 2 μM, 5 μM and 1 μM, respectively, were administered in DMSO, and the maximum concentration of the vehicle did not exceed 0.05% (v/v). This concentration of vehicle was without effect on any of the parameters measured (data not shown). Inhibitors were added to the culture medium 60 min before rtTNFα stimulation and maintained throughout the 18 h treatment with rtTNFα. Cycloheximide (CHX) (Sigma) was applied
at a concentration of 10 μg/ml to serum-starved cells for 30 min before rtTNFα stimulation. Conditioned medium (see below) was applied directly to cells plated in 12-well plates for 24 h, in a volume of 1 ml/well, after repeatedly washing the cells with DMEM to remove the growth medium.

Glucose uptake measurements in trout muscle cells

Determination of 2-deoxyglucose (2-DG) uptake in trout muscle cells was performed as previously described (19, 21). Cells were washed twice with PBS and were incubated with HEPES buffered saline containing 50 μM 2-deoxyglucose (0.5 μCi/ml 2-[3H]-DG) for 30 min at 18ºC. Cells were subsequently rinsed three times with ice-cold PBS solution containing 50 mM D-glucose. Finally, cells were lysed with 0.05 N NaOH and lysates were counted with scintillation liquid in a β-counter (Packard Bioscience, Meriden, CT). Nonspecific uptake was carried out in the presence of cytochalasin B (50 μM) during the assay, and these values were subtracted from all other values. Protein concentration was measured by the Bradford method (7). Glucose uptake was measured in triplicate, normalised to total protein and expressed as fold change with respect to unstimulated cells.

L6 cell culture

In this study we also used the rat skeletal muscle cell line L6 that stably expresses brown trout GLUT4 harbouring an exofacial myc epitope (btGLUT4myc) (19). L6-btGLUT4myc myoblasts were maintained in α-MEM supplemented with 10% FBS, blasticidin S (2 μg/ml) and 1% antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B) in a humidified atmosphere of air and 5% CO₂ at 37ºC. Myoblasts were obtained by seeding L6 cells in medium containing
10% FBS and used at confluence, 2 days after seeding. Myotubes were obtained by differentiating L6 cells in medium supplemented with 2% FBS within 6-7 days after seeding.

**Determination of the proportion of btGLUT4myc at the cell surface**

Cell surface levels of trout GLUT4 in L6 muscle cells stably expressing GLUT4myc were quantified by a colorimetric method, as previously described (19, 40, 46). In brief, L6-btGLUT4myc cells were stimulated with rhTNFα in serum-deprived medium (α-MEM 0.1% FBS) for 18 h. Medium was then removed by washing (3X) in ice cold PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ at 4°C (PBS+, pH 7.4). To label cell surface btGLUT4myc, cells were blocked in 5% goat serum (GS) in PBS+ for 15 min, and then incubated with α-myc antibody solution (1.0 µg/ml in PBS+ with 5% GS) for 1 h at 4°C. Following labeling, excess anti-myc antibodies were removed by extensive washing in ice-cold PBS+. Cells were then fixed in 4% paraformaldehyde in PBS+ for 30 min and quenched in 100 mM glycine in PBS+ for 10 min at 4°C. Cell surface GLUT4-bound anti-myc antibodies were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies followed by detection of bound HRP by o-phenylenediamide assay, as previously described (40, 46). The fraction of btGLUT4myc at the cell surface, measured in triplicate, was expressed as fold induction with respect to unstimulated cells and normalized to total protein.

**Trout myoblast viability assays**

Myoblast viability after rtTNFα treatment was assayed by propidium iodide (PI) (Sigma) staining and analyzed by fluorescence-activated cell sorter (FACS). Briefly, trout muscle cells at day 2 of culture were trypsinised, washed with PBS, stained with
PI by resuspending the cells in PBS containing 50 μg/ml PI (pH = 8.3), treated with RNase (Sigma) for 30 min at 37 ºC and incubated with PI solution for 15 min at room temperature. Viability of the samples was determined after 5000-6000 events in a flow cytometer.

*Generation of conditioned media from rainbow trout macrophages*

Rainbow trout macrophages were isolated from the head kidney as previously described (34) with some modifications (39). Briefly, adult rainbow trout were anesthetized in 3-aminobenzoic acid ethyl ester (0.1 g/l), animals were sacrificed by a blow to the head and the head kidney was dissected. The tissue was homogenized using 100 μm nylon mesh cell-strainers in the presence of DMEM medium containing high glucose, 10% FBS and 100 μg/ml Primocin. Cell homogenates were plated on 6-well culture plates previously coated with poly-L-lysine and maintained at 16 ºC under 5% CO₂. Non-adhering cells were removed after 24 h and new medium was added. The cells were incubated for 4-5 additional days until they had acquired typical cytological characteristics of differentiated macrophages (34) and, before stimulation, macrophages were incubated in serum-free medium for 3 h. The control conditioned medium (indicated as CM-control), was collected from cells incubated in DMEM 0% FBS, and the lipopolysaccharide (LPS)-stimulated conditioned medium (indicated as CM-LPS), was collected from cells incubated in DMEM medium 0% FBS and stimulated for 12 h with 50 μg/ml LPS. After 12 h, CM-control and CM-LPS media were collected and centrifuged at 600 x g for 15 min at 4ºC to eliminate cell debris. In order to remove LPS, CM-LPS was incubated with polymyxin B (Affi-Prep Polymyxin Affinity Support, BioRad, Barcelona) for 4 h at 4ºC (indicated as CM-LPS-PMX).
**Statistical analysis**

Values are given as mean ± SE. Analysis of differences between groups was performed by the Mann–Whitney U-test or unpaired Student’s t-test and were considered statistically significant when \( P < 0.05 \).

**Results**

*rtTNFα stimulates glucose uptake in trout myoblasts and myotubes*

We examined the effects of rtTNFα on glucose uptake in trout muscle cells at day 2 (Fig 1A) and at day 10 (Fig 1B) in culture, which represent myoblast and myotube stages, respectively (12). We incubated trout muscle cells for 24 h in the absence or presence of different doses of rtTNFα and observed that rtTNFα significantly (\( P < 0.05 \)) stimulated glucose uptake in myoblasts, at doses from 10 to 50 ng/ml, and in myotubes, at doses from 1 to 50 ng/ml. The magnitude of the rtTNFα–stimulated glucose uptake was similar in myoblasts and myotubes, approximately two-fold over basal glucose uptake levels (4.20 ± 0.38 and 3.50 ± 0.45 pmol 2-DG/µg protein in myoblasts and myotubes, respectively). Time-course experiments between 3 and 24 h showed that rtTNFα, at a dose of 50 ng/ml, significantly stimulated glucose uptake after 6 h and that the strongest response was after 24 h (Supplementary Figure 1). In order to compare the activity of rtTNFα with that of recombinant human TNFα (rhTNFα), we performed dose-response experiments with the two forms of TNFα on glucose uptake by trout myoblasts. Our results show that rtTNFα significantly stimulated glucose uptake at doses as low as 10 ng/ml, as also illustrated in Figure 1A, but rhTNFα was only active at the highest dose tested (50 ng/ml), indicating that rhTNFα has a lower biological activity (approximately 40%) than rtTNFα in this cell system and when used at the same dose (Supplementary Figure 2).
rtTNFa and insulin do not show additive effects on glucose uptake in trout muscle cells.

Insulin significantly stimulated glucose uptake in trout muscle cells, with higher insulin-stimulated glucose uptake in myotubes (Fig. 2B) over myoblasts (Fig. 2A), as previously shown (21). In myoblasts or myotubes incubated in the presence of different concentrations of rtTNFa (1, 10 and 50 ng/ml), insulin did not further stimulate glucose uptake, suggesting that the effects of insulin and rtTNFa are not additive (Fig. 2).

rtTNFa does not affect cellular viability in trout muscle cells

Due to the known pro-apoptotic actions of TNFα, we performed viability assays in rainbow trout myoblasts incubated for 24h in the absence or presence of different concentrations of rtTNFa (1, 10 and 50 ng/ml). Our results suggest that rtTNFa does not affect the viability of the cultured trout muscle cells (Fig. 3).

The cell surface levels of trout GLUT4 increase in response to TNFa

In order to investigate if the stimulation of glucose uptake by TNFa was due to changes in the cell surface levels of trout GLUT4, as previously shown for rat GLUT4 (40), we used L6 cells stably expressing trout GLUT4 with an exofacial myc epitope (L6-btGLUT4myc) (19) and stimulated them with rhTNFa at the myoblast and myotube stages (Fig.4). Our results indicate that rhTNFa increased the cell surface levels of trout GLUT4 in L6-btGLUT4myc myoblasts at concentrations as low as 1 ng/ml (Fig. 4A). In L6-btGLUT4myc myotubes, rhTNFa significantly increased btGLUT4myc cell surface levels at all concentrations tested (1, 10 and 50 ng/ml) (Fig. 4B).

Inhibitors of NF-κB, p38 and JNK signalling pathways impair rtTNFa-stimulated glucose uptake
Next, we conducted experiments to determine if the stimulatory effects of rtTNFα on glucose uptake by trout muscle cells could be mediated by the p38MAPK, NF-κB or JNK signalling pathways, known to be activated by TNFα (4) and shown to participate in the TNFα stimulation of glucose uptake and rat GLUT4 cell surface levels in L6 cells (40). In order to do this, we incubated rainbow trout myoblasts with rtTNFα (50 ng/ml) in the absence or presence of BAY 11-7082 (1 μM), SB220025 (2 μM) or SP600125 (5 μM), specific inhibitors of the NF-κB, p38MAPK and JNK signalling pathways, respectively, and performed glucose uptake assays. The results obtained indicate that all three inhibitors significantly (P < 0.05) abrogated the stimulatory effects of rtTNFα on glucose uptake in trout myoblasts (Fig. 5). For BAY 11-7082 and SB220025, rtTNFα-stimulated glucose uptake was reduced below the control levels. None of the three inhibitors significantly affected basal glucose uptake levels (data not shown). These results indirectly suggest that rtTNFα may stimulate glucose uptake by trout muscle cells via the activation of the p38MAPK, NF-κB and JNK signalling pathways.

*Conditioned medium from LPS-stimulated trout macrophages increases glucose uptake in trout myoblasts*

We have previously shown that rainbow trout macrophages respond to bacterial LPS by strongly inducing TNFα mRNA expression (34, 39) and secretion into the medium (39). To examine whether TNFα and other cytokines secreted by trout macrophages stimulated with LPS affect glucose uptake in trout myoblasts, we incubated cells with conditioned medium from non-stimulated (CM-Control) and LPS-stimulated trout macrophages (CM-LPS) for 24 h. Our results indicate that CM-LPS significantly stimulated (P < 0.05) glucose uptake over the control medium (CM-Control) (Fig. 6A). Incubation of trout myoblasts with CM-LPS treated with polymyxin B to remove
residual LPS (CM-LPS+PMX) elicited a similar stimulation of glucose uptake than when cells were incubated with CM-LPS alone. This result suggests that glucose uptake is likely stimulated by macrophage-released factors and not by the presence of residual LPS in the medium. To confirm the presence of native TNFα in CM-LPS media we performed Western blotting using a polyclonal antibody against rainbow trout TNFα. As previously shown (39), CM-LPS is enriched with native trout TNFα when compared to CM-Control (Fig. 6B); however, cytokine levels could not be quantified due to the lack of quantitative measuring techniques.

* Cycloheximide impairs rtTNFα-stimulated glucose uptake in trout myoblasts 

We used the protein biosynthesis inhibitor cycloheximide (CHX) to investigate if de novo protein synthesis is required for the stimulation of glucose uptake by rtTNFα in trout myoblasts. Cells were serum starved and preincubated with CHX (10 μg/ml) for 30 min, followed by a 24 h incubation with rtTNFα (50 ng/ml). At this dose, CHX did not affect cell viability (data not shown). Our results indicate that CHX completely blocked the stimulatory effects of rtTNFα on glucose uptake without affecting basal uptake (Fig. 7).

* Discussion 

In the mammalian skeletal muscle, pro-inflammatory cytokines such as TNFα can exert a variety of actions that range from physiological (e.g. cell proliferation and myogenic differentiation) to pathological (e.g. insulin resistance and cachexia) depending on cytokine production levels. It is well known that high levels of TNFα produced during inflammation cause insulin resistance and chronically elevated TNFα levels are associated with inflammatory diseases, sepsis and cancer (31). However, there is
increasing evidence that cytokines and particularly those produced in skeletal muscle (i.e. myokines), TNFα and IL-6 being among the best characterized, can play an important physiological role during muscle development and function (31, 37). In fish, as in other non-mammalian vertebrates, the effects of cytokines on skeletal muscle have not been investigated to date. In the present study, we provide evidence for the first time on the ability of trout TNFα of recombinant origin to stimulate glucose uptake by trout myoblasts and myotubes in culture without affecting cell viability. Our results on the stimulatory effects of low concentrations of TNFα (as low as 1 ng/ml) on glucose uptake by trout muscle cells are in agreement with those from other studies using mammalian muscle cells and mammalian cytokines at comparable concentrations (14, 40, 48). This, together with the lack of change in cell viability, suggests that the effective concentrations of rtTNFα may indeed be physiological, although no methods are currently available, as indicated above, for measuring the levels of TNFα produced during physiological and pathological conditions. In addition, rtTNFα did not show synergistic or additive effects with insulin on glucose uptake by trout muscle cells. Recent data on L6 cells indicated that TNFα does not lead to an activation of the insulin-signaling pathway, ruling out the possibility that insulin and TNFα may exert their biological effects through the same pathway (40). Instead, we believe that the lack of additivity of rtTNFα and insulin may be due to the ability of rtTNFα to maximally stimulate glucose uptake and trout GLUT4 translocation, as evidenced by the similar magnitude of increase of these parameters by rtTNFα and insulin (in this and in Diaz et al. (19, 21)).

Making use of a previously developed L6 cell line stably expressing trout GLUT4 with an exofacial myc epitope that has been a useful tool to study the traffic characteristics of
trout GLUT4 (3, 19), we have also demonstrated for the first time that trout GLUT4 can translocate to the plasma membrane from intracellular stores in response to TNFα stimulation. Although this last evidence was generated using a heterologous system, previous studies from our group reporting on the stimulation of glucose uptake and on the traffic of trout GLUT4 by insulin using this same system (19) would support the hypothesis that trout TNFα may stimulate glucose uptake in trout muscle cells by stimulating the translocation of trout GLUT4 to the plasma membrane. In fact, mammalian TNFα has the same stimulatory effect on GLUT4 cell surface levels in L6 cells (40).

The rtTNFα-induced increase in glucose uptake by trout muscle cells was mimicked by CM-LPS, shown in this (Fig. 6B) and other studies (39) to be enriched in TNFα. This result suggests that macrophage-derived native trout TNFα has the same biological effects as rtTNFα. However, it is important to point out that other soluble macrophage-derived factors in addition to TNFα may have also contributed to the stimulatory effect of CM-LPS on glucose uptake since LPS is known to directly induce the expression of a large repertoire of immune mediators in trout macrophages (33). A recent study using mammalian cells has identified interleukin-10 (IL-10) as a potential macrophage-derived insulin-sensitizing cytokine in skeletal muscle cells (43); however, it is not known if trout macrophages express IL-10 in response to LPS treatment or if IL-10 could have direct effects on trout muscle cells. To ensure that the observed results with CM-LPS were due to the secreted cytokine(s) and not affected by the presence of LPS, we removed LPS from CM-LPS by treatment with polymyxin B. Trout muscle cells incubated with polymyxin-treated CM-LPS took up glucose at similar levels as cells incubated with non-treated CM-LPS, evidencing that the increase in glucose uptake in
trout muscle cells incubated with CM-LPS is due to macrophage-secreted cytokines and not to residual LPS in the medium. This conclusion is supported by recent data indicating that LPS does not affect glucose uptake, GLUT4 cell surface levels nor activation of insulin signalling molecules in mammalian muscle cells (43).

It is well known that TNFα is a pleiotropic cytokine that signals through diverse signalling pathways (4). In mammalian skeletal muscle cells, TNFα stimulates glucose uptake by activating three important signalling pathways: NF-κB, p38MAPK and JNK (40). In the present study, to investigate the signalling pathways involved in the stimulatory action of rtTNFα on glucose uptake by trout muscle cells we used BAY 11-7082, SB220025 and SP600125, specific inhibitors of the NF-κB, p38MAPK and JNK pathways, respectively. Our results on the similar sensitivity of rtTNFα stimulation to these inhibitors as that of human TNFα in L6 cells (40) with regard to glucose uptake suggest that rtTNFα may require the implication of the NF-κB, p38MAPK and JNK pathways to stimulate glucose uptake in trout muscle cells. The possibility that these pathways may be activated by rtTNFα in trout muscle cells is supported by recent data showing the activation of the NF-κB, p38MAPK and JNK pathways by human TNFα in L6 cells (40). In addition to providing information on the signalling pathways used by rtTNFα, these results provide further evidence for the ability of trout muscle cells to respond to this cytokine. In contrast to mammals, in which TNFα acts by binding to two different types of TNFα receptors (TNFR1 and TNFR2) (44), nothing is known on the type(s) of TNFα receptors present in trout skeletal muscle cells. In this regard, our attempts to detect binding of radiolabeled rhTNFα in trout muscle cells were unsuccessful (Vraskou and Planas, unpublished observations), suggesting that rhTNFα is probably not well recognized by the trout TNFα receptor(s). This conclusion is also
supported by our observation on the significantly lower ability of rhTNFα, when compared to rtTNFα, to stimulate glucose uptake by trout muscle cells. The difference in biological action of human and trout TNFα in trout muscle cells is expected due to the low degree of similarity at the amino acid sequence level (approx. 30%) between piscine and mammalian TNFα molecules (6, 25, 50) and argues against the use of mammalian TNFα for studies using fish species, particularly when homologous cytokines are available.

In this study we also show that the stimulation of glucose uptake by rtTNFα in trout muscle cells after a 24 h incubation is dependent on de novo protein synthesis. Based on the similar stimulatory effects of rtTNFα and insulin (19, 21) on glucose uptake and on the reported induction of glucose transporter (GLUT4 and GLUT1) mRNA and protein levels by insulin in trout skeletal muscle cells and tissues (19-21), we hypothesize that rtTNFα could stimulate glucose uptake by increasing GLUT4 cell surface levels and also by regulating glucose transporter expression. This hypothesis is consistent with published data showing that TNFα increases glucose uptake and GLUT1 protein levels at the plasma membrane in mammalian skeletal muscle cells (14). Future studies in our laboratory will be devoted to investigate the regulation of glucose transporter expression by rtTNFα in trout skeletal muscle.

*Perspectives and Significance*

In the present study we provide evidence, for the first time in non mammalian vertebrates, for the stimulatory effect of homologous TNFα on glucose uptake by trout skeletal muscle cells in culture. These results suggest that the ability of muscle cells to respond to TNFα by increasing the cell surface levels of GLUT4 and, consequently,
their ability to take up glucose has been retained during evolution from fish to mammals. Furthermore, our results support the notion that TNFα is an important physiological factor contributing to the regulation of skeletal muscle function in vertebrates. In view of the strong induction of TNFα production in trout macrophages by immune stimuli (e.g. LPS), it is tempting to speculate that infectious processes caused by bacterial pathogens could have important metabolic effects in fish skeletal muscle. Indeed, pathogen-induced changes in skeletal muscle metabolism that are mediated by cytokines (e.g. TNFα) could have an impact in production of fish species in aquaculture. However, important basic questions regarding TNFα action in skeletal muscle in fish remain to be answered, namely (1) can TNFα in fish, like in mammals (42), be produced by skeletal muscle cells (i.e. myocytes) and therefore act in a autocrine/paracrine fashion?, (2) what are the factors regulating the expression and production of TNFα? and (3) what is the nature, binding characteristics and regulation of receptor(s) for TNFα in trout skeletal muscle? Answers to these questions will shed light onto the physiological role of TNFα in skeletal muscle in vertebrates.
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Figure Legends

Figure 1. Dose-related effects of rtTNFα on glucose uptake in rainbow trout myoblasts (A) and myotubes (B). Cells were serum-starved and stimulated in the absence or presence of different concentrations of rtTNFα (1, 10 and 50 ng/ml) for 24 h and 2-DG uptake was subsequently determined. Results are expressed as fold stimulation above the control group, which was set to 1. Results shown are the means ± SE of six independent experiments for myoblasts and five independent experiments for myotubes, each performed in duplicate or in triplicate. Different letters indicate significant differences ($P < 0.05$).

Figure 2. Effects of rtTNFα and insulin on glucose uptake in rainbow trout myoblasts (A) and myotubes (B). Cells were serum-starved and incubated for 24 h in the absence or presence of different concentrations of rtTNFα (1, 10 and 50 ng/ml) for 24 h followed by a 30 min stimulation with insulin (1 μM) and 2-DG uptake was determined. Results are expressed as fold stimulation above the control group, which was set to 1. Data represents the means ± SE of 6 independent experiments with myoblasts and 5 independent experiments with myotubes, each performed in duplicate or triplicate. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 3. Viability of trout myoblasts incubated with rtTNFα. Cells were incubated in the absence or presence of different concentrations of rtTNFα (1, 10 and 50 ng/ml) for 24 h. Cells were resuspended in PBS containing 50 μg/ml propidium iodide and the viability was analysed in a flow cytometer in 5000-6000 events.
Figure 4. Effects of TNFα on cell surface levels of trout GLUT4myc in L6 myoblasts (A) and myotubes (B). The proportion of trout GLUT4myc at the cell surface relative to control was determined in L6 cells stably expressing trout GLUT4myc and stimulated with TNFα (0, 1, 10, or 50 ng/ml) for 24 h, as described in Material and Methods. Results shown are the means ± SE of four independent experiments, each performed in triplicate. Results are expressed as fold stimulation above the control group, which was set to 1. Different letters indicate significant differences ($P < 0.05$).

Figure 5. Effects of specific inhibitors of the NF-κB (BAY 11-7082), p38MAPK (SB220025) and JNK (SP600125) pathways on rtTNFα-stimulated glucose uptake by rainbow trout myoblasts. Cells were pre-treated with BAY 11-7082 (1 μM), SB220025 (2 μM) or SP600125 (5 μM) for 60 min before rtTNFα stimulation (50 ng/ml for 24 h). After the incubation period, 2-DG uptake assay was performed as indicated in Material and Methods. Results shown are the mean ± SE of four independent experiments, each performed in triplicate and are expressed relative to the control group, which was set to 1. Different letters indicate significant differences ($P < 0.05$).

Figure 6. Effects of conditioned medium from lipopolysaccharide (LPS)-stimulated trout macrophages on glucose uptake in rainbow trout myoblasts. In A) the effects on glucose uptake are shown. Cells were incubated in control conditioned medium, in LPS-conditioned medium or in LPS-conditioned medium treated with polymyxin B (LPS+PMX conditioned medium) for 24 h. After the incubation period, 2-DG uptake assay was performed. Results shown are the mean ± SE of three independent experiments, each performed in triplicate and are expressed relative to the control group, which was set to 1. Different letters indicate significant differences ($P < 0.05$).
B), the presence of native trout TNFα in control and LPS conditioned media from trout macrophages was evaluated by Western blotting and the results are expressed in arbitrary units of optical density (OD) and shown as the mean ± SE from four independent experiments. A representative Western blot is shown in the inset graph.

**Figure 7.** Effects of cycloheximide (CHX) on rtTNFα-stimulated glucose uptake by rainbow trout myoblasts. Cells were pretreated with CHX (10 μg/ml) for 60 min before rtTNFα stimulation (50 ng/ml for 24 h). After the incubation period, 2-DG uptake assay was performed. Results shown are the mean ± SE of four independent experiments, each performed in triplicate and are expressed relative to the control group, which was set to 1. Different letters indicate significant differences ($P < 0.05$).
Figure 1

(A) 2-DG uptake (Fold stimulation) as a function of rtTNFα (ng/ml) concentration.

(B) 2-DG uptake (Fold stimulation) as a function of rtTNFα (ng/ml) concentration.
Figure 2

A

**2-DG uptake (Fold stimulation)**

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B

**2-DG uptake (Fold stimulation)**

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Figure 3

The bar chart shows the percentage of viable and non-viable cells as a function of increasing concentrations of rtTNFa (ng/ml). The x-axis represents the concentration levels (0, 1, 10, 50 ng/ml), while the y-axis represents the percentage of cells. The black bars indicate viable cells, and the white bars represent non-viable cells. Error bars are included to indicate the variability in the data.
Figure 4

A

![Bar chart showing cell surface GLUT4 (Fold stimulation) with rhTNFα (ng/ml) as the x-axis and cell surface GLUT4 as the y-axis. Bars are labeled with letters 'a', 'b', and 'ab' for different groups.](image)

B

![Bar chart showing cell surface GLUT4 (Fold stimulation) with rhTNFα (ng/ml) as the x-axis and cell surface GLUT4 as the y-axis. Bars are labeled with letters 'a', 'b', and 'ab' for different groups.](image)
Figure 5

2-DG uptake (Fold change over control)

<table>
<thead>
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<th>Treatment</th>
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<th>b</th>
<th>c</th>
<th>c</th>
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</table>
Figure 6

A

2-DG uptake (Fold stimulation)

0.0
0.5
1.0
1.5
2.0
2.5
3.0

Control
LPS
LPS+PMX

Conditioned medium

B

Macrophage-secreted TNFα (ODs)

0
50
100
150
200

Control
LPS

Conditioned medium
Figure 7

![Bar graph showing 2-DG Uptake (Fold Change over Control) with conditions for rtTNFα and CHX]

- rtTNFα: - \(-
  + \(+\)
- CHX: - \(-
  + \(+\)

Values marked with different letters (a, b) indicate statistically significant differences.