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RESEARCH ARTICLE

Adiponectin effects and gene expression in rainbow trout: an *in vivo* and *in vitro* approach

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SUMMARY

Here we present the presence of adiponectin and adiponectin receptors [type 1 (adipoR1) and type 2 (adipoR2)] in rainbow trout (*Oncorhynchus mykiss*) tissues and cell cultures together with the response to different scenarios. In response to fasting, adiponectin expression was up-regulated in adipose tissue, while the expression of its receptors increased in white and red muscle. Insulin injection decreased adipoR1 expression in white and red muscles. We deduce that the adipoRs in trout muscle show opposite responses to increasing insulin plasma levels, which may maintain sensitivity to insulin in this tissue. Adiponectin expression was inhibited by the inflammatory effect of lipopolysaccharide (LPS) in adipose tissue and red muscle. Moreover, results indicate that LPS may lead to mobilization of fat reserves, increasing adipoR1 expression in adipose tissue. The effects of LPS could be mediated through tumour necrosis factor α (TNFα), at least in red muscle. Insulin, growth hormone and TNFα all diminished expression of adipoR2 in adipocytes and adipoR1 in myotubes, while insulin increased the expression of adipoR2 in the muscle cells. Adiponectin activates Akt in rainbow trout myotubes, which may lead to an increase in fatty acid uptake and oxidation. Overall, our results show that the adiponectin system responds differently to various physiological challenges and that it is hormonally controlled *in vivo* and *in vitro*. To the best of our knowledge, this is the first time this has been demonstrated in teleosts, and it may be a valuable contribution to our understanding of adipokines in fish.

Key words: muscle cell, adipose cell, insulin, growth hormone, lipopolysaccharide, tumour necrosis factor α, TNFα, fatty acid uptake, fatty acid distribution, fatty acid oxidation, PI3K/Akt, MAPK, AMPK, signalling pathways.

INTRODUCTION

Over the past few years, a new role for adipose tissue as an endocrine organ participating in the regulation of energy homeostasis has been established and analysed extensively in mammals in relation to insulin resistance, obesity and the regulation of fatty acid (FA) and glucose metabolism. Nevertheless, there is little information available about adipokines in lower vertebrates such as fish. Although the presence of leptin and tumour necrosis factor α (TNFα) has been described in various fish species (Kling et al., 2009; Saera-Vila et al., 2007), adiponectin has been identified in zebra fish (Nishio et al., 2008), and only very recently in rainbow trout (Kondo et al., 2011).

The most abundant adipokine in mammalian plasma is adiponectin (Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996). Adiponectin is a member of the complement 1q family which, in circulation, exists as a full-length or smaller globular domain (gAd) (Waki et al., 2003). The plasmatic levels of adiponectin have been negatively correlated with obesity, insulin resistance, metabolic syndrome and cardiovascular diseases (Kadowaki et al., 2006). It has been suggested that the anti-inflammatory action of adiponectin could be a possible link between all these disorders (Fantuzzi, 2008), although the mechanisms remain to be determined. Adiponectin binds to membrane receptors: the adiponectin receptor 1 (adipoR1) and adiponectin receptor 2 (adipoR2). These are seven transmembrane domain proteins, which are structurally and functionally distinct from G protein-coupled receptors (Yamauchi et al., 2003a). Adiponectin activates the AMP-activated protein kinase (AMPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt but not the MAPK (p42/44 MAPK) signalling pathways in muscle (Yamauchi et al., 2003a; Lee et al., 2008); after this activation, adiponectin exerts its functions, modulating insulin sensitivity, glucose homeostasis and lipid metabolism. Previous studies have shown that this leads to increased glucose and FA uptake and FA oxidation in rat muscle and muscle cells (Ceddia et al., 2005; Mullen et al., 2009).

Some fish species, including salmonids, have adaptations to fasting in their lipid and glucose metabolism (Navarro and Gutiérrez, 1995; Albalat et al., 2006) since they have a fasting period in their normal annual life cycle, either because of spawning migration or periods of low food availability, which also alters the plasma hormonal levels of insulin, insulin-like growth factor (IGF)-I and growth hormone (Gutiérrez et al., 2006). Little information exists on the changes in adipokine levels with food deprivation in fish (Kling et al., 2009).

A reduction in the expression of adiponectin in adipose tissue was found after a fasting period, while the expression of adipoR1 increased in the muscle of mice (Bertile and Raclot, 2004; Tsuchida et al., 2004) and in the liver of zebra fish (Nishio et al., 2008).

The regulation of *adiponectin* and *adipoR* gene expression is complex and is affected by some of the most important metabolism

regulatory factors. For instance, insulin seems to be a positive regulator of adiponectin gene expression and secretion in 3T3-L1 adipocytes (Pereira and Draznin, 2005), although the regulation of *adiponectin* by insulin is not completely understood. Moreover, humans with obesity-associated insulin resistance have lower levels of adiponectin in circulation (Ryo et al., 2004). Additionally, adipocytes are considered to be integral components of the immune system that respond actively to infection and inflammatory mediators, such as lipopolysaccharide (LPS) and TNFα. Adiponectin is described as an anti-inflammatory agent that reinforces the role of adipose tissue in the response to infection and inflammation (Fasshauer et al., 2004; Ajuwon et al., 2009).

The aim of this study was to analyse the response of the trout adiponectin and adipoR system to different challenges, including fasting, hormone and inflammatory agent treatments. We hypothesized that muscle and adipose tissue can be organs of adiponectin expression and target tissues at the same time, with a role in the regulation of trout metabolism. We analysed how expression of *adiponectin* and its receptors is regulated by nutritional status and by insulin, growth hormone and inflammatory mediators in trout muscle and adipose tissue, through a combination of *in vivo* and *in vitro* studies. Finally, we also examined the effects of adiponectin in muscle metabolism.

Our results demonstrated not only that feeding conditions regulate *adiponectin* and the expression of its receptors in a tissue-specific manner in rainbow trout but also that insulin, among others, is a regulator of the adipokine system *in vivo* and *in vitro*. We also observed that adiponectin activates the PI3K/Akt signalling pathway in muscle cells of rainbow trout in culture, provoking an increase in FA uptake and oxidation.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) were obtained from the fish farm 'Truites del Segre' (Lleida, Spain) and acclimatized to laboratory conditions at the facilities of the Faculty of Biology of the University of Barcelona, in a closed-water flow system at a constant temperature of 14±1°C, for 15 days before any experiment was conducted. Animals were maintained in a 12 h:12 h light:dark cycle and fed a total of 1% of their body mass twice a day with a standard commercial diet (DibaqAquatex, Segovia, Spain). All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the legislation and procedures established by the EU and by the Spanish and Catalan government.

Adiponectin, adipoR1 and adipoR2 tissue distribution

After the acclimatization period, five rainbow trout (approximately 70 g) were killed by a quick blow to the head. Several tissue samples were extracted to analyse adiponectin, *adipoR1* and *adipoR2* gene expression. Animals were fasted for 16 h before sampling.

Fasting experiment

The animals (70 ± 6.5 g) were fed or starved for 15, 25 and 35 days. Animals (N=5 for each group) were killed by a quick blow to the head, and white muscle, red muscle and adipose tissue were extracted, frozen in liquid nitrogen and stored at –80°C until analysis. Fed control animals were fasted for 16 h before sampling.

Injection experiment

Animals with an average mass of 176.30±5.97 g were injected intraperitoneally (1 µl g^{-1} body mass) with vehicle (phosphate buffer saline, PBS) as a control, recombinant human insulin (21.6 pmol g^{-1} body mass, Sigma-Aldrich, Madrid, Spain, ≥27.5 units mg^{-1}) (Plisetskaya et al., 1985), recombinant human TNFα (1 ng g^{-1} body mass, Sigma-Aldrich) or LPS (*E. coli*, serotype O26:B6, at 6 µg g^{-1} body mass, Sigma-Aldrich) (Albalat et al., 2005c). Animals were fasted for 16 h before the beginning of the experiment. After 4, 12 and 24 h post-injection, five animals from each treatment group were killed by a quick blow to the head, and white and red muscle and adipose tissue were extracted, frozen in liquid nitrogen and stored at –80°C until analysis.

Cell culture procedure

Myocyte cells were isolated, pooled and cultured from rainbow trout of approximately 10 g as described previously (Castillo et al., 2002) and allowed to differentiate until day 11 (myotube stage). Pre-adipocyte cell culture was carried out and differentiated *in vitro* as previously reported (Bouraoui et al., 2008). Samples for RNA extraction were taken at different development stages of myocytes [days 2 (myocytes), 6 (myoblasts) and 11 (myotubes) of culture] and pre-adipocytes [days 8 (when the differentiation cocktail was added to the cell medium), 15 (early differentiated adipocytes) and 22 (fully differentiated adipocytes) of culture]. Myocytes at day 11 (myotube stage) were starved for 4 h in serum-free medium and stimulated with the indicated doses of hormones for different times, and the RNA was then extracted. The mature adipocyte isolation was carried out as reported by Albalat and co-workers (Albalat et al., 2005b). After 6 h of incubation with insulin (1 µmol l^{-1}), recombinant salmon/trout growth hormone (GH) (GroPep, Berlin, Germany) (10 nmol l^{-1}) or TNFα (100 ng ml^{-1}), the RNA was isolated.

RNA isolation and cDNA synthesis

Total RNA from trout tissues and cells was extracted using the TriReagent method according to the manufacturer's instructions (Ambion, Applied Biosystems, Madrid, Spain). The quantity and quality of isolated RNA was determined by spectrophotometry with ND-1000 Nanodrop (Labtech International Ltd, Ringmer, UK). For cDNA synthesis, 3 µg of RNA for tissues or 1 µg for cells, 3 µl of a blend 2:1 random hexamers (600 µmol l^{-1})/oligo dT (50 µmol l^{-1}), 2 µl dNTP (10 mmol l^{-1}), 0.5 µl reverse transcriptase (20 U µl^{-1}) and 0.5 µl of RNAse inhibitor (40 U µl^{-1}) were mixed with the kit buffer in a final volume of 20 µl (Transcriptor first strand cDNA synthesis kit, Roche, Berlin, Germany), and incubated at 50°C for 60 min. The enzymes were then inactivated at 85°C for 5 min.

Real-time PCR analysis

PCR measurements were performed by applying the primers at 0.5 µmol l^{-1} with one-fortieth of the cDNA synthesis reaction and SYBR-Green PCR mix (Bio-Rad, Madrid, Spain) in a total volume of 20 µl. The Q-PCR primer sequences for the target genes (*adiponectin*, *adipoR1* and *adipoR2*) and reference genes (*eF1α*, *18S*, *β-actin*) are shown in Table 1. The primers for the *adiponectin*, *adipoR1* and *adipoR2* were designed on the expressed sequence tags (EST) from the database 'Computational Biology and Functional Genomes Laboratory' (http://compbio.dfci.harvard.edu/tgi/). Reactions were performed in an iQCycler IQ Real-time Detection System (Bio-Rad). Each PCR product was sequenced to confirm identity and all were found to be 100% identical to the respective sequence. Twofold serial dilutions of total RNA were made for efficiency calculations. Reactions were performed in duplicate and the fluorescence data acquired during the extension phase were

Table 1. Rainbow trout primer sequences used for real-time PCR

Gene	Database	Accession number	Amplicon size (bp)	Annealing temperature (°C)	Direction	Primer sequence
Adiponectin	DFCI	TC167753	165	62	F	AGCCCGTCATGTTCACCTAC
					R	GAAGGTGGAGTCGTTGGTGT
AdipoR1	DFCI	TC165904	199	60	F	TCCACTCCCACCAGATCTTC
					R	CGTGTTCCAGCAGCACTTTA
AdipoR2	DFCI	TC140134	196	60	F	CTGATCATGGGCTCCTTTGT
					R	ACACCACTCAGACCCAGACC
FATP1	DFCI	CA373015	157	60	F	AGGAGAGAACGTCTCCACCA
					R	CGCATCACAGTCAAATGTCC
CD36	DFCI	AY606034	106	62	F	CAAGTCAGCGACAAACCAGA
					R	ACTTCTGAGCCTCCACAGGA
LPL	GenBank	AJ224693	164	50	F	TAATTGGCTGCAGAAAACAC
					R	CGTCAGCAAACTCAAAGGT
HSL	DFCI	TC138452	175	58	F	AGGGTCAATGGTCATCGTCTC
					R	CTTGACGGAGGGACAGCTAC
PPARα	GenBank	AY494835	195	54	F	CTGGAGCTGGATGACAGTGA
					R	GGCAAGTTTTTGCAGCAGAT
PPARβ	GenBank	AY356399.1	195	60	F	CTGGAGCTGGATGACAGTGA
					R	GTCAGCCATCTTGTTGAGCA
PPARγ	DFCI	CA345564	171	60	F	GACGGCGGGTCAGTACTTTA
					R	ATGCTCTTGGCGAACTCTGT
eF1α	GenBank	AF498320	159	58	F	TCCTCTTGGTCGTTTCGCTG
					R	ACCCGAGGGACATCCTGTG
18S	GenBank	TC158109	189	64	F	GGCGCCCCCTCGATGCTCTTA
					R	CCCCCGGCCGTCCCTCTTAAT
β-*Actin*	GenBank	AJ438158	112	61	F	ATCCTGACGGAGCGCGGTTACAGC
					R	TGCCCATCTCCTGCTCAAAGTCCA

F, forward primer; R, reverse primer.

normalized to the indicated reference gene using the delta-delta method (Livak and Schmittgen, 2001).

Western blot analysis

At day 4 of differentiation, the myocyte cells were starved for 4 h, pre-stimulated with inhibitors [(1 μmol l^{-1} wortmannin (Sigma-Aldrich) and 50 μmol l^{-1} PD98059 (Sigma-Aldrich)] for 30 min when necessary and subsequently incubated with 2.5 μg ml^{-1} globular adiponectin (gAd) or 1 μmol l^{-1} insulin for 30 min. The medium for the control cells was maintained without inhibitors or peptides. The cells were washed twice with ice-cold PBS and lysed with ice-cold lysis buffer (1% NP-40, 10 mmol l^{-1} Tris, 140 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA, pH 7.6) containing a protease inhibitor cocktail (at a dilution of 1/200, Sigma-Aldrich) and phosphatase inhibitors (50 mmol l^{-1} NaF and 0.4 mmol l^{-1} sodium orthovanadate). Protein (30 μg) was loaded in a 12% polyacrylamide gel to perform electrophoresis (SDS-PAGE). Polyvinylidene fluoride (PVDF) membranes were incubated overnight at 4°C with antibodies for the MAPK, Akt and their phosphorylated forms [anti-p42MAPK (catalogue number 9107), anti-phospho-p44/42 MAPK (catalogue number 9106), anti-Akt (catalogue number 9272) and anti-Akt-P (catalogue number 9271), Cell Signaling Technology Inc., Beverly, MA, USA] at a dilution of 1:1000. Secondary antibodies linked to horseradish peroxidase (HRP) were added for 1 h at room temperature, and immunoreactive bands were visualized by enhanced chemical luminescence (ECL) and quantified with an image analyser (Totallab v.1.00, Nonlinear Dynamics Ltd, Durham, NC, USA, 2000).

Glucose and FA uptake assays in muscle cells

For the glucose and FA uptake assays, rainbow trout muscle cells (day 11, myotube stage) were incubated for 4 h with Dulbecco's modified Eagle medium (DMEM) without fetal bovine serum (FBS). After this period they were incubated (60 min) with DMEM in the presence or absence of human globular adiponectin (BioVision, San Francisco, CA, USA) at 2.5 μg ml^{-1}. Glucose uptake was measured as previously described using D-[U-^{14}C]-labelled glucose as radioactive tracer (Codina et al., 2008). FA uptake was performed using medium containing 30 μmol l^{-1} total oleic acid and 0.3 μCi ml^{-1} of [1-^{14}C]-labelled oleic acid (NEC 317050UC, Perkin Elmer Corp., Foster City, CA, USA). Oleic acid, in its salt form, was bound to BSA-FA free at a ratio of 2.7:1. In both cases, an aliquot of the lysate was kept for protein determination using the Bradford method (Bradford, 1976).

FA distribution assays

The effect of adiponectin (2.5 μg ml^{-1}) on oleic acid distribution was studied in 11-day-old myotubes, as reported previously for rainbow trout myocytes (Sánchez-Gurmaches et al., 2010). After 2 days of incubation, radioactivity incorporated in CO_2, acid-soluble products (ASP) and main lipid classes were quantified.

Statistical analysis

In vitro experiments were performed at least in triplicate. For the *in vivo* experiments, N=5 was used. The t-test, one-way or two-way ANOVA, followed by Tukey's test were used when necessary, as

indicated in the figure captions. Differences were considered statistically significant when $P<0.05$.

RESULTS

Tissue distribution

The highest mRNA expression of *adiponectin* was found in red muscle tissue followed by the white muscle, and it was expressed to a much lesser degree in adipose tissue (Fig. 1A). *AdipoR1* was most strongly expressed in the spleen and heart of rainbow trout, and weaker expression was also found in the remaining tissue types (Fig. 1B). In contrast, *adipoR2* was strongly expressed in proximal intestine and pyloric caeca (Fig. 1C), although the levels were not significantly different from the rest of the tissues.

Effect of fasting on *adiponectin*, *adipoR1* and *adipoR2* gene expression

In both white and red muscles, fasting affected the expression of *adiponectin* and its receptors. While *adiponectin* gene expression was reduced (at 15 days of fasting in white muscle but not until 35 days of fasting in red muscle), the expression of its two receptors increased with the extension of the fasting period in white muscle (Fig. 2A–F). In contrast, fasting increased the expression of *adiponectin* in adipose tissue after 15 and 35 days (Fig. 2G). However, *adipoR1* expression did not change significantly throughout the study (Fig. 2H). The expression of *adipoR2* in adipose tissue diminished after 35 days of fasting (Fig. 2I).

Effects of insulin, TNFα and LPS injection on *adiponectin*, *adipoR1* and *adipoR2* gene expression

The effects of insulin, TNFα and LPS injection were analysed in white and red muscles and adipose tissue after 4, 12 and 24 h (Fig. 3). When insulin was injected, it reduced the expression of *adipoR1* in both muscles and in adipose tissue (Fig. 3B,E,H); these effects occurred at different times after the injection of insulin. *AdipoR2* expression decreased quickly in the adipose tissue (Fig. 3D). TNFα caused a down-regulation of *adiponectin* expression in red muscle. *AdipoR1* gene expression responded similarly in this tissue, while in the white muscle it increased (Fig. 3B,E). No effects of TNFα on the expression of the *adipoR2* were observed. Fish injected with LPS showed a reduced expression of *adiponectin* in red muscle and adipose tissue (Fig. 3A,G). Reduced expression of *adipoR1* was also observed in white and red muscles in the fish injected with LPS, whereas it increased in adipose tissue (Fig. 3B,E,H). No changes in the expression of *adipoR2* were observed.

A summary of the changes in gene expression from *in vivo* experiments is shown in Table 2.

Adiponectin, *adipoR1* and *adipoR2* gene expression in adipose cells

The expression of *adiponectin* decreased with the differentiation of pre-adipocyte cells (day 8) into adipocytes (days 15 and 22). In contrast, both of its receptors increased their expression (Fig. 4A–C). The effect of insulin ($1\,\mu mol\,l^{-1}$), GH ($100\,nmol\,l^{-1}$) and TNFα ($100\,ng\,l^{-1}$) was evaluated in mature freshly isolated adipocytes of rainbow trout. Although a 2.5- and 1.5-fold increase in *adiponectin* gene expression was observed after GH and insulin stimulation, respectively, no significant changes were found (Fig. 4D). Nevertheless, GH significantly decreased the expression of *adipoR1* and *adipoR2* (Fig. 4E,F). Insulin and TNFα stimulation also reduced expression of the latter.

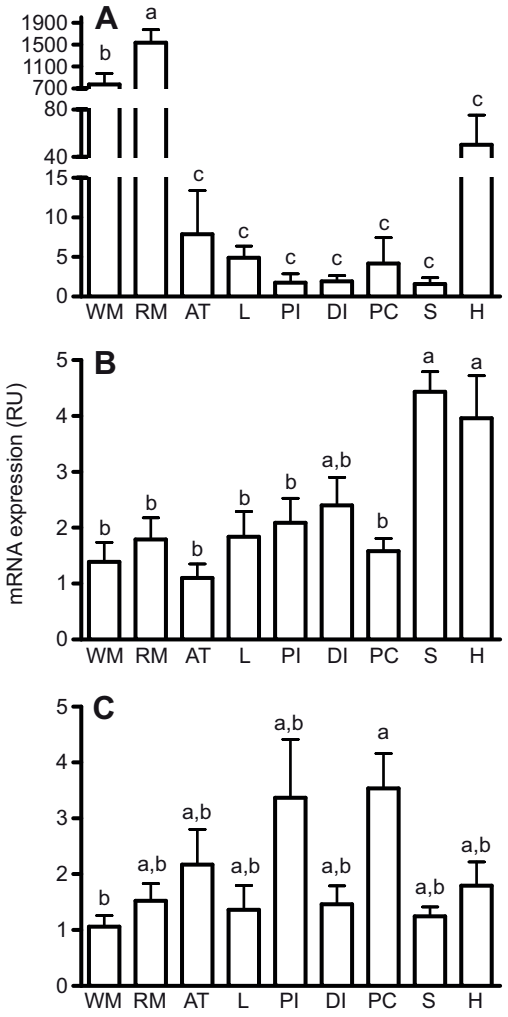

Fig. 1. mRNA expression profile of *adiponectin* (A), *adipoR1* (B) and *adipoR2* (C) in rainbow trout tissues. *18S* gene expression was used as the reference gene. Values are expressed as means ± s.e.m. (*N*=5) (RU, relative units). The tissue with the lowest expression rate was set at 1. Statistically significant differences are denoted by different letters. WM, white muscle; RM, red muscle; AT, adipose tissue; L, liver; PI, proximal intestine; DI, distal intestine; PC, pyloric caeca; S, spleen; H, heart.

Adiponectin, *adipoR1* and *adipoR2* mRNA expression in muscle cells

A clear reduction in *adiponectin* mRNA expression was found in the developmental stages of the skeletal muscle cells of rainbow trout, which was significant in myotubes (day 11 of differentiation; Fig. 5A). Myotube stage cells were treated with insulin and GH for 3, 6 and 18 h, and with TNFα for 24 h. Only TNFα affected the expression of *adiponectin* in myotubes of rainbow trout, inducing a decrease in its expression (Fig. 5D). Different regulation on the adiponectin receptors by insulin was found: for example, while insulin decreased the expression of *adipoR1*, the expression of *adipoR2* was increased (Fig. 5E,F). Also, GH and TNFα treatments down-regulated the expression of *adipoR1* after 18 h, but no effects on *adipoR2* gene expression were found after incubation with these two hormones.

Adiponectin signalling pathways in skeletal muscle cells

Adiponectin signalling pathways were analysed in 4-day-old skeletal muscle cells in culture. Adiponectin effectively activated Akt

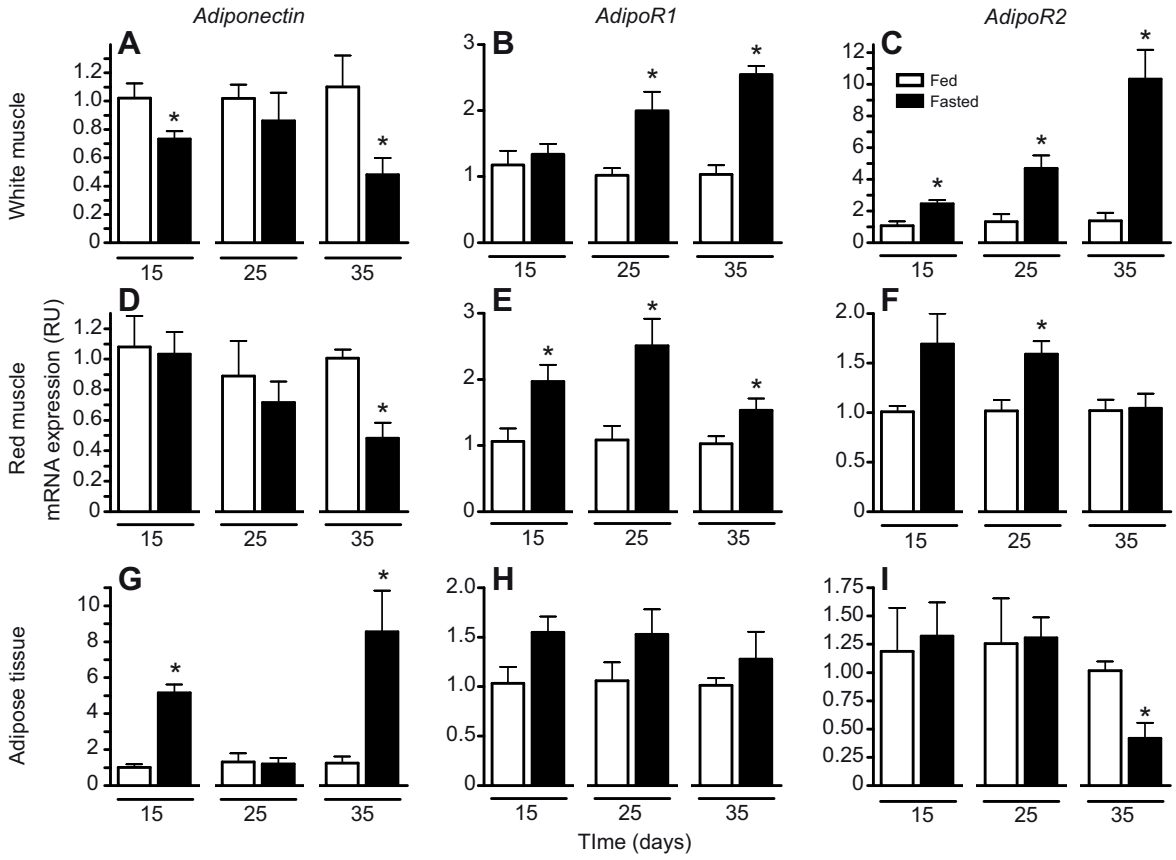

Fig. 2. Fasting effects on *adiponectin*, *adipoR1* and *adipoR2* gene expression in white muscle (A–C), red muscle (D–F) and adipose tissue (G–I) in rainbow trout. Fish were fed (white bars) or fasted (black bars) for 15, 25 or 35 days prior to sampling. *eF1α* gene expression was used as the reference gene. Values are expressed as means ± s.e.m. (*N*=5) (RU, relative units). Asterisk (*) indicates significant differences between fed and fasted rainbow trouts ($P<0.05$, *t*-test).

phosphorylation and it was only inhibited by pre-incubation with wortmannin (Fig. 6A). Adiponectin was ineffective in activating the MAPK signalling pathway, but the previously PD98059-treated cells contained a lower amount of phospho-MAPK than the cells treated only with adiponectin (Fig. 6B).

Adiponectin effects on glucose and FA uptake and FA distribution

In skeletal muscle cells of rainbow trout in culture, pre-incubation with adiponectin ($2.5\,\mu g\,ml^{-1}$) for 1 h did not affect the glucose or oleate uptake rates (data not shown). However, increased FA oxidation, reported as CO_2 production, was found after 2 days with adiponectin and the labelled oleate (Fig. 7A). Treatment with adiponectin did not affect ASP production in rainbow trout skeletal muscle cells in culture (Fig. 7B) but a significant reduction in the FA in the medium was observed together with a slight, but not significant, increase in the presence of triacylglycerols inside the cells (Fig. 7C).

Adiponectin effects on mRNA expression in rainbow trout myotubes

Adiponectin stimulation ($2.5\,\mu g\ ml^{-1}$) reduced the expression of lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) after 6 and 3 h, respectively (Fig. 8C,D). The mRNA levels of expression of *adipoR1* and peroxisome proliferator activated receptor (*PPARα*) were also decreased by this stimulation (Fig. 8F,H).

DISCUSSION

The vegetable ingredients and the high level of lipids found in the diets of fish maintained in aquaculture can lead to high lipid deposition in the fillet and as peri-visceral adipose tissue. This accumulation can result in production losses, alteration of flesh quality and possible adverse effects on fish health, although these remain to be established (Seierstad et al., 2005; Saera-Vila et al., 2009; Seierstad et al., 2009). For these reasons and also because of the importance of fish lipids in the human diet, the regulation of lipid metabolism has become a notable point of interest in aquaculture research (Drevon, 1992). Nevertheless, the understanding of endocrine control of fish lipid metabolism is poor and the role of adipokines, including adiponectin, is even more so.

As is the case in zebrafish (Nishio et al., 2008), and the very recent study performed in rainbow trout (Kondo et al., 2011), we have also observed that the expression of adiponectin in muscles is higher than in adipose tissue. In addition, adiponectin mRNA expression was two times higher in the oxidative red muscle than in the glycolytic white muscle, which corresponds with previous studies assessing different types of fibres in mice (Krause et al., 2008). Nevertheless, a higher expression of adiponectin in adipose tissue in comparison with muscle was found in mammalian species. It appears that a high expression of adiponectin in muscle could be a characteristic feature in fish. However, we still do not know whether or not the peptide is produced and released to the circulation and could be considered a myokine (Pedersen and Febbraio, 2008;

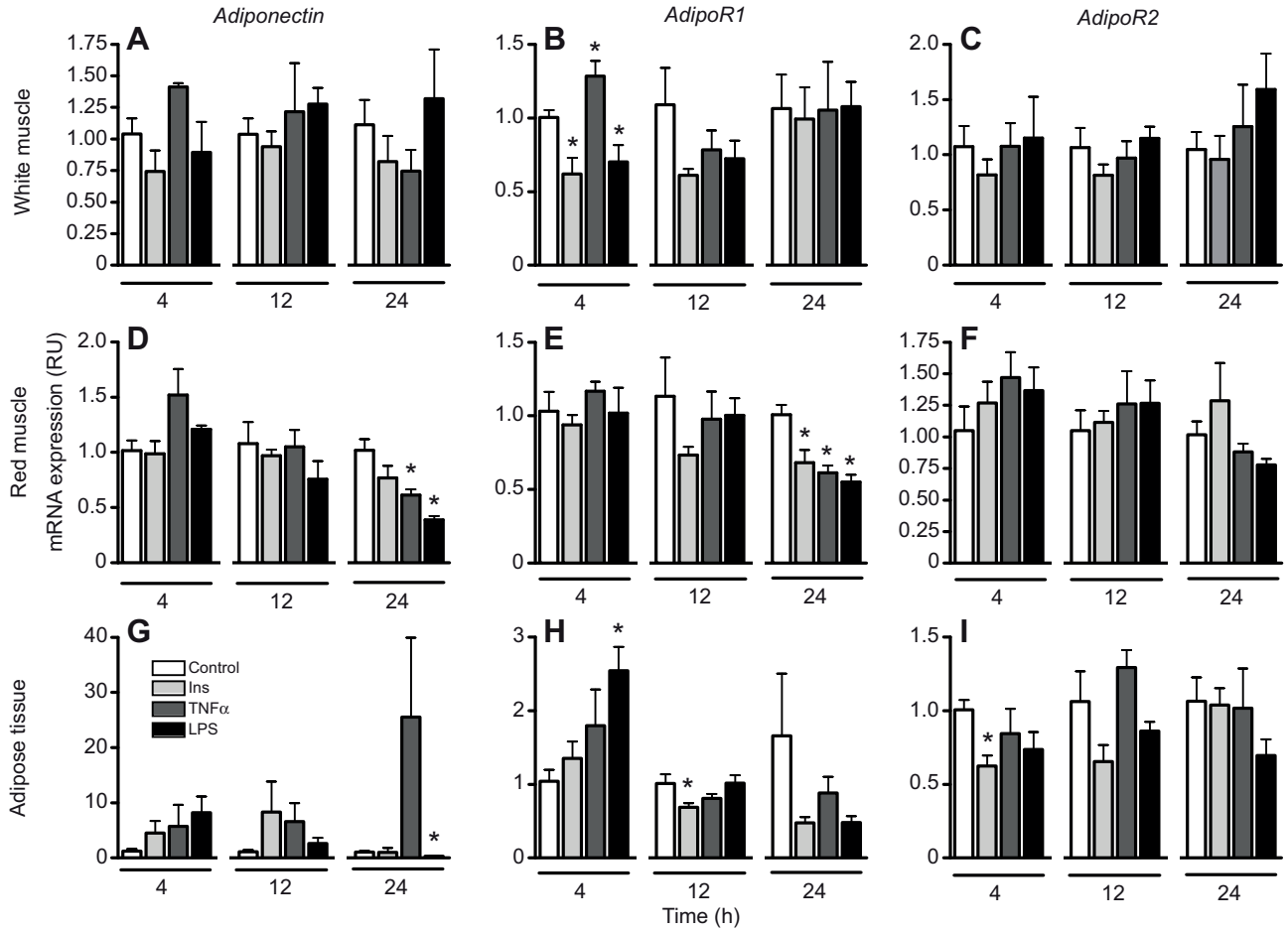

Fig. 3. Insulin, TNFα and LPS injection effects on *adiponectin*, *adipoR1* and *adipoR2* gene expression in white muscle (A–C), red muscle (D–F) and adipose tissue (G–I) in rainbow trout. Fish were injected intraperitoneally (1 μl g^{-1} body weight) either with a vehicle (PBS) as a control, insulin (Ins) (21.6 pmol g^{-1} body weight), TNFα (1 ng g^{-1} body weight) or LPS (*E. coli*, serotype O26:B6, at 6 μg g^{-1} body weight) and samples were taken at 4, 12 and 24 h following treatment. *eF1α* gene expression was used as a reference gene. Values are expressed as means ± s.e.m. (*N*=5) (RU, relative units). Asterisk (*) indicates significant differences between treated and control rainbow trouts (*P*<0.05, *t*-test).

Walsh, 2009). Regarding adiponectin receptors, in humans and mice adipoR1 is mainly located in muscle and adipoR2 in liver, but this tissue-specific location is not present in other species such as pigs, where a more wide distribution of adiponectin receptors is found. This is particularly true in zebra fish (Nishio et al., 2008) and in rainbow trout (present study), where the adipoRs are widely distributed among different tissues and both receptors can be considered ubiquitous.

In vivo experiments

Fasting is a catabolic situation that promotes the mobilization of lipid reserves from adipose tissue to other organs (Navarro and Gutiérrez, 1995). The most remarkable change observed during fasting in trout was the inverse relationship between the regulation of adiponectin and its receptors in skeletal muscle. Although adiponectin mRNA levels decreased in both muscles, the expression of its receptors increased, especially adipoR2 in white muscle (up to 10-fold increase). As a result of this opposite regulation, the adiponectin system could probably be directed to maintain the level of muscular fatty acid oxidation. The availability of fatty acids during food deprivation comes from the increased adipose tissue lipolytic activity (Albalat et al., 2005b). In this sense, since adiponectin correlates with lipolytic activity (Bulló et al., 2005), the 8-fold induction of adiponectin expression in trout adipose tissue with fasting may promote lipid mobilization in an autocrine or paracrine way, even though the expression of adipoR2 decreased in this tissue. Nevertheless, the effects of adiponectin on lipolysis are controversial

Table 2. Summary of the changes in gene expression from *in vivo* experiments

	White muscle	Red muscle	Adipose tissue
Fast			
Adiponectin	↓	↓	↑↑
AdipoR1	↑	↑	–
AdipoR2	↑↑	↑	↓
Insulin			
Adiponectin	–	–	–
AdipoR1	↑	↓	↓
AdipoR2	–	–	↓
TNFα			
Adiponectin	–	↓	–
AdipoR1	↑	↓	–
AdipoR2	–	–	–
LPS			
Adiponectin	–	↓	↓
AdipoR1	↓	↓	↑
AdipoR2	–	–	–

↓ and ↑ indicate decrease and increase in gene expression, respectively, and – indicates that there was no change.

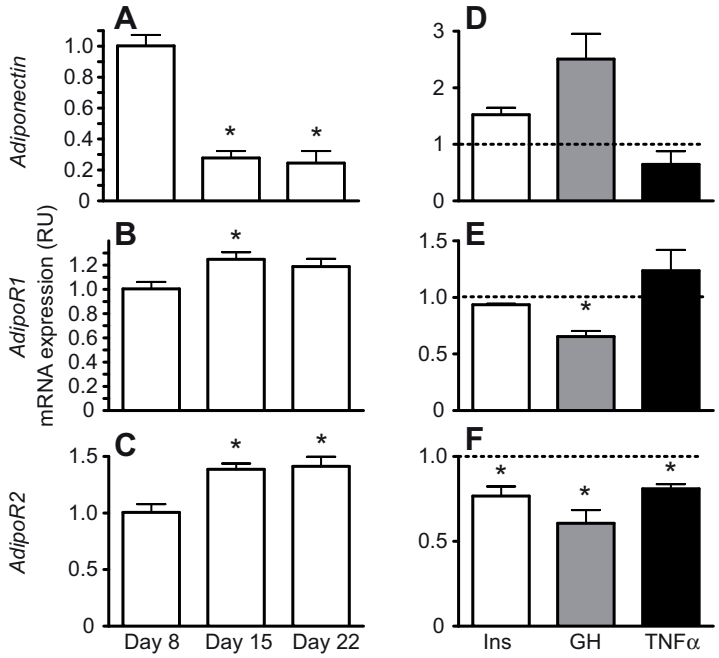

Fig. 4. Adipocyte differentiation and insulin (Ins), GH and TNFα effects on *adiponectin*, *adipoR1* and *adipoR2* mRNA expression in rainbow trout adipocytes. Pre-adipocyte cells (day 8), early differentiated adipocytes (day 15) and fully differentiated adipocytes (day 22) were used to assess the adipocyte differentiation profile expression of *adiponectin* (A), *adipoR1* (B) and *adipoR2* (C) using β-actin as the reference gene. Values are expressed as means ± s.e.m. (*N*=3) (RU, relative units). Asterisk (*) indicates significant differences from cells at day 8 (*P*<0.05, *t*-test). Mature isolated adipocytes were stimulated with insulin (1 μmol l^{-1}), GH (100 nmol l^{-1}) and TNFα (100 ng ml^{-1}) for 6 h, and after this *adiponectin* (D), *adipoR1* (E) and *adipoR2* (F) mRNA expression were determined using *eF1α* gene expression as a reference gene and the control (not shown) was set to 1. Values are expressed as means ± s.e.m. (*N*=3). Asterisk (*) indicates significant differences between control and treated adipocytes (*P*<0.05, *t*-test).

since recent studies have reported anti-lipolytic actions of adiponectin in mammalian adipocytes (Wedellova et al., 2010; Qiao et al., 2011). Further studies of adiponectin effects on trout adipocytes would help to understand its metabolic regulation.

The administration of insulin decreased adipoR1 expression in rainbow trout white and red muscles, which is a response opposite to the changes observed during fasting, when circulating insulin levels are low (Navarro and Gutiérrez, 1995). Nevertheless, the adiponectin muscular mRNA levels were not affected by insulin. Taken together, expression of trout adiponectin and its receptors was slightly modified by insulin injection in both muscles and adipose tissue, which suggests the absence of a close relationship between plasma insulin and the adiponectin system, as occurs in mammals (Pereira and Draznin, 2005).

Adipose tissue has important functions in the immune response, although they have not been fully assessed in fish. The Gram-negative bacteria toxin LPS has many effects on lipid metabolism in fish, although these responses are not always the same as in

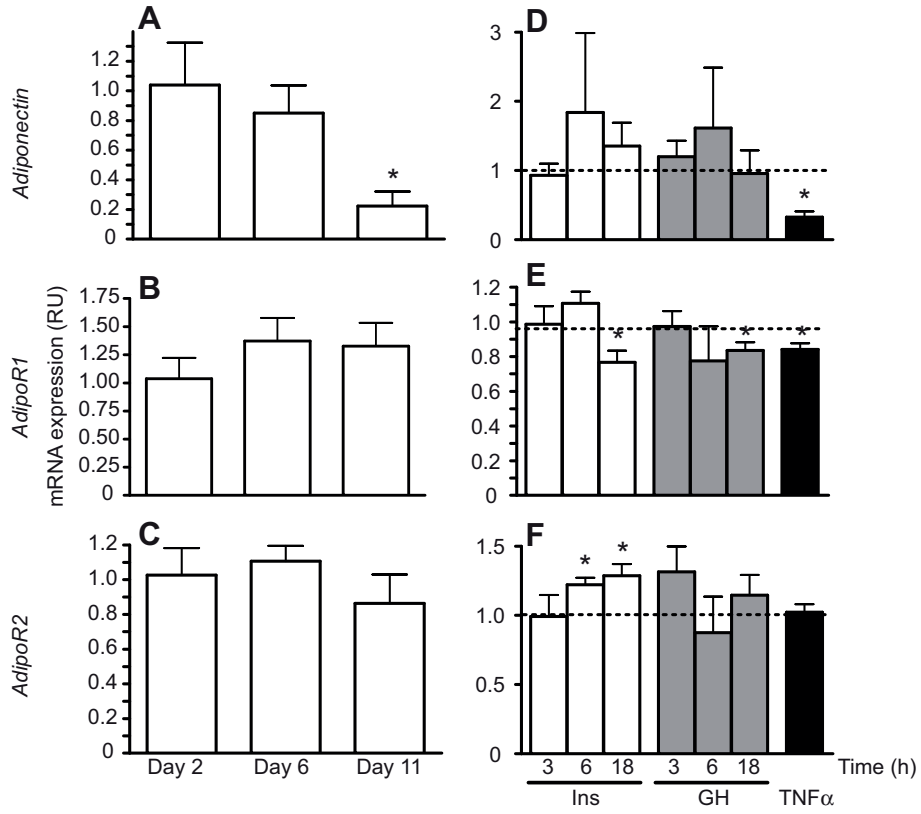

Fig. 5. Myocyte differentiation and insulin (Ins), GH and TNFα effects on *adiponectin*, *adipoR1* and *adipoR2* mRNA expression in rainbow trout myotubes. Myocytes (day 2), myoblast (day 6) and myotube (day 11) samples were used to assess the myogenic differentiation profile expression of *adiponectin* (A), *adipoR1* (B) and *adipoR2* (C) using *eF1α* gene expression as the reference gene. Values are expressed as means ± s.e.m. (*N*=3) (RU, relative units). Asterisk (*) indicates significant differences from cells at day 2 (*P*<0.05, *t*-test). Rainbow trout myotubes were stimulated with insulin (1 μmol l^{-1}), and GH (100 nmol l^{-1}) for 3, 6 and 18 h, and with TNFα (100 ng ml^{-1}) for 24 h, and after this *adiponectin* (D), *adipoR1* (E) and *adipoR2* (F) mRNA expression were determined using *eF1α* gene expression as a reference gene and the control (not shown) was set to 1. Values are expressed as means ± s.e.m. (*N*=3). Asterisk (*) indicates significant differences between control and treated myotubes (*P*<0.05, *t*-test).

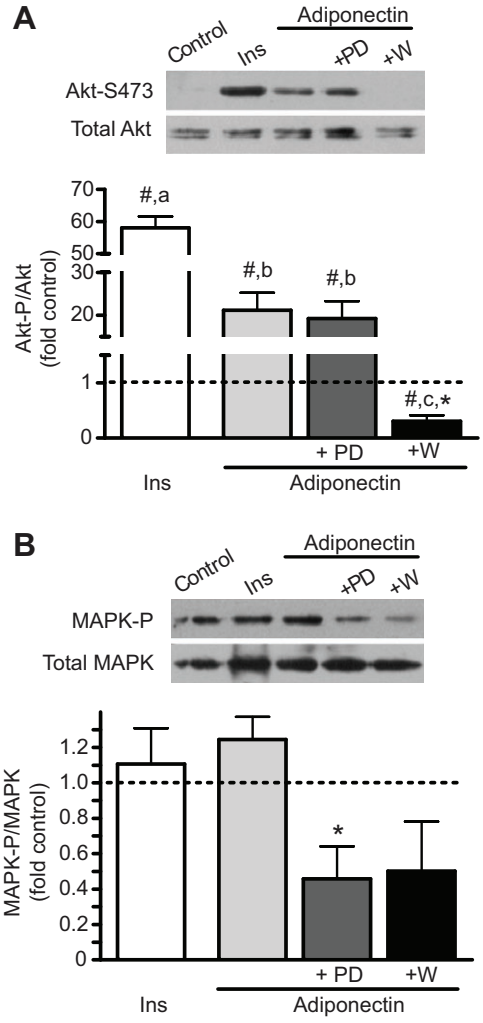

Fig. 6. Adiponectin effect on PI3K/Akt and MAPK signalling pathways in rainbow trout myocytes. Cells were incubated with inhibitors (wortmannin (W) at 1 μmol l^{-1} or PD98059 (PD) at 50 μmol l^{-1}) for 30 min if necessary and after this with adiponectin (2.5 μg ml^{-1}) or insulin (Ins, 1 μmol l^{-1}) for 30 min to assess the effects on the activation of intracellular signalling pathways [Akt (A) and MAPK (B)]. Values are expressed as means ± s.e.m. (*N*=3). Values of >1 or <1 indicate an increase or decrease with respect to control values (not shown). Values not sharing letters are significantly different and values showing an '#' are significantly different from control values ($P<0.05$, ANOVA followed by Tukey's test). Asterisk (*) indicates significant differences of the inhibitor pre-stimulated cells and adiponectin-treated myocytes ($P<0.05$, *t*-test).

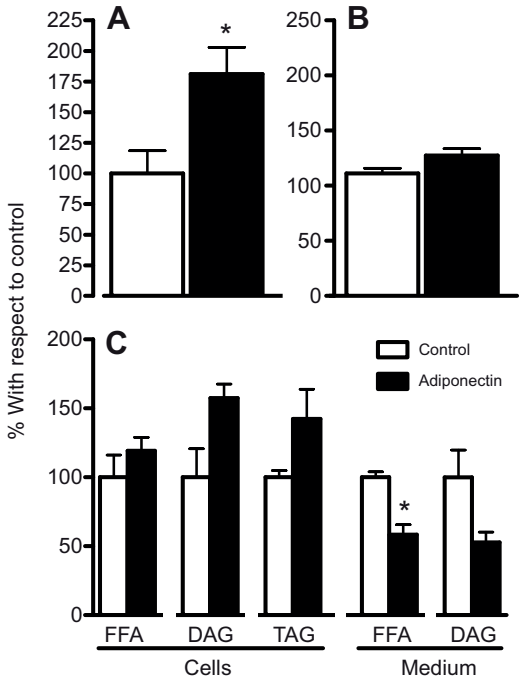

Fig. 7. Adiponectin effects on the FA distribution and oxidation in rainbow trout myocytes. Rainbow trout myotubes (day 11) were incubated for 2 days with [1-^{14}C]-labelled oleate with or without adiponectin (2.5 μg ml^{-1}) and CO_2 (A) and ASP (B) production and the main lipid compartments (C) determined as explained in the Materials and methods section. Values are expressed as means ± s.e.m. (*N*=3). Asterisk (*) indicates significant differences between control and adiponectin-treated myotubes ($P<0.05$, *t*-test). FFA, free fatty acids; DAG, diacylglycerols; TAG, triacylglycerols.

mammals (Swain et al., 2008). It has been reported that LPS injection in mammals does not affect adiponectin expression in adipose tissue (Gomez-Ambrosi et al., 2004; Jacobi et al., 2004). In our experiment, the first response of adipose tissue was to increase the expression of adipoR1, which could indicate an increased mobilization of the lipid reserves to other tissues with immune function, such as spleen or head kidney.

Some authors suggest that TNFα mediates many of the effects of LPS, although these effects are not identical (Goetz et al., 2004; Iliev et al., 2005). In mammals, TNFα administration *in vivo*, as an inflammatory stimulus, reduced the plasma adiponectin levels and the production of this adipokine in adipose tissue (Kappes and Loffler, 2000; Fasshauer et al., 2002; Bruun et al., 2003; Degawa-Yamauchi et al., 2005), leading to positive feedback and inflammation (Fantuzzi, 2008). Although we have not found differences in the expression of mRNA in adipose tissue, adiponectin expression in red muscle tissue, which is strongest in rainbow trout, was reduced by TNFα injection, which may promote the same vicious cycle described above. Nevertheless, the known anti-inflammatory action of adiponectin still has to be demonstrated in fish. It is also remarkable that the different regulation of adipoR1 between white and red muscles by TNFα could be related to the metabolic differences between these two kinds of muscle *in vivo* (Johnston, 1981). Interestingly, adipoR1 expression decreased in the red muscle tissue of fish injected with both LPS and TNFα, which suggests that LPS acts through TNFα in this tissue by decreasing its sensitivity to adiponectin and therefore decreasing energy consumption. Nevertheless, LPS could also act through other cytokines such as IL-1 or IL-6 (Castellana et al., 2008) and their actions could explain the different regulation of adipoR1 expression observed between TNFα and LPS in white muscle tissue. These results indicate that the short-term regulation of the trout adiponectin system may be important during the acute phase response, and may relate to the modulation of metabolism and immune response.

In vitro experiments

Adiponectin expression increases during adipocyte differentiation in mammals (Scherer et al., 1995). Furthermore, adiponectin is able to induce muscle gene expression and cell differentiation in C2C12 myoblasts, and the peptide is produced in cultured cells (Scherer et al., 1995; Fiaschi et al., 2009). Nevertheless, we found a decrease

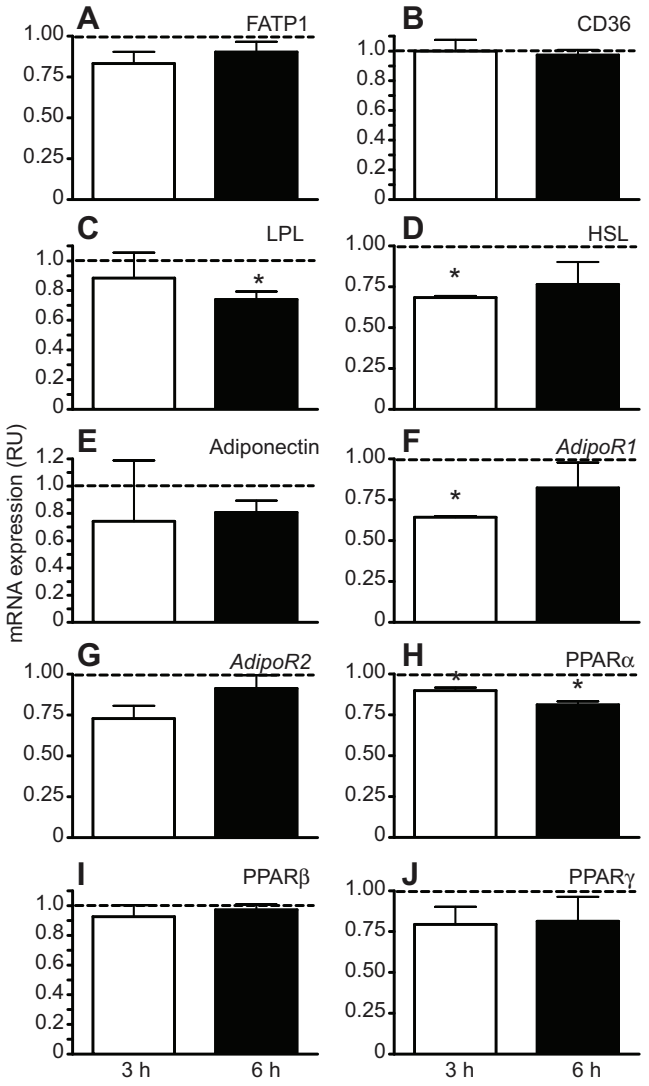

Fig. 8. Adiponectin effects on the mRNA expression of rainbow trout myotubes. Rainbow trout myotubes (day 11) were incubated for 3 h (white bars) or 6 h (black bars) with adiponectin (2.5 μg ml^{-1}) prior to mRNA extraction. The mRNA expression of the target genes was normalized using *eF1α* as a reference gene, and the control (not shown) was set to 1. Values are expressed as means ± s.e.m. (*N*=3) (RU, relative units). Asterisk (*) indicates significant differences between control and treated myotubes (*P*<0.05, *t*-test).

in adiponectin expression during trout pre-adipocyte and myocyte differentiation. However, the increased expression of both adipoRs with the differentiation of trout adipocytes and the absence of changes during myocyte development are consistent with previous studies in mouse cell lines (Fasshauer et al., 2004; Fiaschi et al., 2009). These observations suggest that, in trout, the adiponectin system might play a more relevant role in adipocyte differentiation than in myocyte maturation. These differences in expression patterns might help to distinguish these two models of cell differentiation towards mature adipocyte and myocyte, respectively, in trout, although further studies are needed.

The effects of hormones on gene expression in adipocyte and myocyte cultured cells were not very pronounced. Regarding GH effects on adipocytes, a decrease in expression of both receptors was observed, suggesting an inhibition of adiponectin actions by this hormone, which might be related, somehow, with the known metabolic actions of GH in fish adipocytes, such as the activation of lipolysis (Albalat et al., 2005a) (M. Monroy, L.C.-G., J.S.-G., E. Capilla, J.G. and I.N., unpublished observations).

The effects of insulin on adipoR1 and adipoR2 in rainbow trout myocyte cell culture differed, with the former decreasing and the latter increasing. Injection of insulin also decreased adipoR1 receptor in both types of muscle *in vivo*, but adipoR2 expression was not modified. A reduction in adiponectin and adipoR1 receptor expression after TNFα incubation in myocytes was also observed in red muscle after peptide injection, suggesting a similar role of this cytokine in *in vitro* and *in vivo* systems. Nevertheless, it has to be taken into account that the two experimental models are very different and not strictly comparable.

Due in part to the high expression of adiponectin found in trout muscle we decided to study possible signalling pathways and effects of adiponectin in trout muscle cells in culture. In fact, the signalling pathways regulated by adiponectin are just beginning to be characterized in mammalian species. It is considered that adiponectin effects are mediated, at least in part, *via* AMPK and PI3K/Akt pathways in mammalian muscle cell models, provoking increases in glucose and FA uptake and oxidation, among others (Yamauchi et al., 2002; Ceddia et al., 2005; Palanivel et al., 2007; Fiaschi et al., 2009). Adiponectin activated the p44/42 MAPK pathway in vascular smooth muscle tissue but it was not observed in C2C12 cells (Yamauchi et al., 2003a). In trout myocytes, it was revealed that adiponectin activates the PI3K/Akt pathway but not the p44/42 MAPK pathway, similar to the process that occurs in C2C12 myocytes. These observations indicate that the conservation of the signalling pathways of adiponectin along the vertebrates does occur, as previously reported for insulin, and also that IGF signalling pathways are conserved in myocytes in culture, which was previously described by our group (Montserrat et al., 2007; Codina et al., 2008). In the present study, wortmannin blocked the stimulatory effect of adiponectin on Akt phosphorylation, demonstrating the specificity of the detected molecule. We have previously described that the PI3K/Akt pathway is involved in some metabolic effects of insulin and IGFs in rainbow trout myocytes in culture, such as increasing the glucose uptake (Codina et al., 2008). However, the specific functions of each receptor, the pathways implicated in adiponectin effects and, moreover, whether or not adiponectin activates AMPK in fish remain to be clarified.

Under our experimental conditions, no changes in the glucose and FA uptake were found after adiponectin treatment of trout muscle cells over a short period; however, after 2 days of incubation with adiponectin, a reduced presence of FA in the medium was observed, which can be associated with the increased FA uptake, as reported elsewhere in rat cardiomyocyte cell cultures (Palanivel et al., 2007). In the same experiments, adiponectin increased FA oxidation, as previously demonstrated for other similar cellular models (Fruebis et al., 2001; Ceddia et al., 2005; Civitarese et al., 2006; Ding et al., 2007), perhaps due to increased carnitine palmitoyltransferase 1 (CPT1) activity (Ding et al., 2007; Li et al., 2007). Although an increased FA oxidation rate is associated with enhanced insulin sensitivity in mammals (Kadowaki et al., 2006), it still has not been tested for fish. Nevertheless, a characteristic marker for FA uptake (the enzyme LPL) decreased its mRNA expression following adiponectin stimulation in rainbow trout myotubes, and the transmembrane FA transporters (FATP1 and CD36) were not affected. This indicates that more research is needed to clarify the mechanisms through which adiponectin increases FA uptake in fish cells. The PPARα, which is related to FA catabolism

in mammals (Leaver et al., 2008), was decreased by adiponectin stimulation, contrary to that found in mice muscle and myocytes, in which both expression and activity of the PPARα increased after adiponectin stimulation (Yamauchi et al., 2001; Yamauchi et al., 2003b). Nevertheless, it is not currently clear whether the PPARs have the same role and specificity in fish. Despite this, it appears that some compensatory effects could be present within the metabolic activation of the FA utilization (CO_2 production) and the down-regulation of the expression of this factor. We have found that adiponectin diminished the expression of adipoR1 but not of adipoR2 in rainbow trout muscle cells, which has been previously observed in human myotubes (McAinch et al., 2006). This effect, together with the strong expression of adiponectin in trout muscle, indicates a regulatory feedback loop where adiponectin may act in an autocrine or paracrine fashion to regulate the function of its receptors.

To our knowledge this is the first time that the effects of endocrine regulation of the adiponectin system have been studied in teleosts. Although more research is needed, we can confirm that the hormones and cytokine studied here (insulin, GH, TNFα), together with fasting and a simulated infection (LPS injection), affect the expression of the adiponectin system, linking adiponectin and its receptors with many physiological challenges in fish. We have also found that adiponectin could activate intracellular signalling pathways and that some of its effects are conserved from fish to mammals.

LIST OF ABBREVIATIONS

adipoR1	adiponectin receptor 1
adipoR2	adiponectin receptor 2
AMPK	AMP-activated protein kinase
ASP	acid-soluble products
FA	fatty acid
GH	growth hormone
HSL	hormone-sensitive lipase
IGF	insulin-like growth factor
LPL	lipoprotein lipase
LPS	lipopolysaccharide
PI3K	phosphatidylinositol 3-kinase
TNFα	tumour necrosis factor α

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