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RESEARCH ARTICLE

Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout (*Oncorhynchus mykiss*)

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SUMMARY

To assess the potential involvement of TORC1 (target of rapamycin complex 1) signalling in the regulation of post-prandial hepatic lipid and glucose metabolism-related gene expression in trout, we employed intraperitoneal administration of rapamycin to achieve an acute inhibition of the TOR pathway. Our results reveal that rapamycin inhibits the phosphorylation of TORC1 and its downstream effectors (S6K1, S6 and 4E-BP1), without affecting Akt and the Akt substrates Forkhead-box Class O1 (FoxO1) and glycogen synthase kinase 3α/β (GSK 3α/β). These results indicate that acute administration of rapamycin in trout leads to the inhibition of TORC1 activation. No effect is observed on the expression of genes involved in gluconeogenesis, glycolysis and fatty acid oxidation, but hepatic TORC1 inhibition results in decreased sterol regulatory element binding protein 1c (SREBP1c) gene expression and suppressed fatty acid synthase (FAS) and glucokinase (GK) at gene expression and activity levels, indicating that FAS and GK activity is controlled at a transcriptional level in a TORC1-dependent manner. This study demonstrates for the first time in fish that post-prandial regulation of hepatic lipogenesis and glucokinase in rainbow trout requires the activation of TORC1 signalling.

Key words: TORC1, rapamycin, fatty acid synthesis, glycolysis, gene expression, rainbow trout.

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INTRODUCTION

The target of rapamycin (TOR) is a central controller of the growth and metabolism of cells in response to nutrients, growth factors and cellular energy status in mammals (Wullschleger et al., 2006). TOR exists in two structurally and functionally distinct complexes, the rapamycin- and nutrient-sensitive TORC1 and the rapamycin- and nutrient-insensitive TORC2 (Kim and Guan, 2011). TORC1 integrates inputs from at least five major intracellular and extracellular cues – growth factors, stress, energy status, oxygen and amino acids – to control many major processes, including protein and lipid synthesis and autophagy (Wullschleger et al., 2006). The mechanism leading to TORC1 activation has already been established in mammals and fish (Kim and Guan, 2011; Lansard et al., 2010; Seiliez et al., 2008). In complex with the intracellular 12 kDa FK506-binding protein (FKBP12), rapamycin binds and inhibits TOR exclusively in TORC1, whereas FKBP12–rapamycin cannot bind TOR in TORC2, making this complex insensitive to direct inhibition by rapamycin at least over short periods (Laplante and Sabatini, 2012; Sarbassov et al., 2004). However, in many cell types (Sarbassov et al., 2005) and *in vivo* studies (Lamming et al., 2012), prolonged rapamycin treatment results in a decrease of TORC2 function, perhaps as a result of depletion of the TORC2 complex (Sarbassov et al., 2006).

It is well known in mammals that TORC1 plays an important role in the control of many aspects of cellular metabolism including lipid metabolism and glucose homeostasis (Wullschleger et al., 2006). TORC1 enhances *de novo* lipogenesis by regulating the expression and processing of sterol regulatory element-binding protein 1c (SREBP1c) (Wan et al., 2011; Yecies et al., 2011), a master regulator of sterol and lipogenic gene transcription (Krycer et al., 2010). Furthermore, TORC1 increases glycolytic flux by activating the transcription and the translation of hypoxia inducible factor 1a (HIF1a) (Düvel et al., 2010), a positive regulator of many glycolytic genes (Gordan et al., 2007).

In contrast, the role of TORC1 in the metabolic response to feeding status or dietary composition in fish has been less well investigated. Rainbow trout have relatively high dietary protein and essential amino acid requirements and poor utilization of dietary carbohydrates compared with humans, rodents and other terrestrial domestic animals (Walton and Cowey, 1982; Wilson, 1994); thus, it represents a suitable model to investigate the involvement of TORC1 in the nutritional control of metabolic gene expression. Most components of the TOR system [TOR, ribosomal protein S6 kinase (S6K1), ribosomal protein S6 (S6) and 4E-binding protein 1 (4E-BP1)] are evolutionarily conserved and have already been characterized in rainbow trout liver and muscle (Lansard et al., 2010; Seiliez et al., 2008; Seiliez et al., 2011a), and viscera and muscle from zebrafish (Seiliez et al., 2013). In fish, activation of the TOR pathway is controlled by re-feeding (Seiliez et al., 2008; Seiliez et al., 2013; Skiba-Cassy et al., 2009) as well as *in vivo* administration of insulin (Plagnes-Juan et al., 2008). As demonstrated using primary culture of rainbow trout hepatocytes, the joint action of insulin and amino acids (particularly leucine) seems to be essential to activate the TOR pathway whereas insulin alone is sufficient to activate protein kinase B (Akt) phosphorylation (Lansard et al., 2011; Lansard et al., 2010).

In rainbow trout, macronutrient composition of the diet affects the TOR signalling pathway and metabolism-related gene

expression. Increasing the dietary proportion of carbohydrates at the expense of proteins impairs activation of the TOR pathway and modifies the expression of several genes related to metabolism (Seiliez et al., 2011a; Skiba-Cassy et al., 2013). Furthermore, an *in vitro* investigation with rainbow trout hepatocytes demonstrated that insulin and amino acids together upregulate lipogenic and glycolytic gene expression in a TOR-dependent manner (Lansard et al., 2010) as observed in mammals (Porstmann et al., 2008). Thus, we put forward the hypothesis that TOR is involved in the post-prandial regulation of hepatic lipid and glucose metabolism-related gene expression in rainbow trout.

To determine the potential involvement of TORC1 signalling in the regulation of post-prandial hepatic metabolism-related gene expression in rainbow trout, we performed intraperitoneal (i.p.) administration of rapamycin. Our goal was to achieve an acute inhibition of the TORC1 signalling pathway *in vivo* and analyse the consequences of this TORC1 inhibition on the expression of several genes related to glucose utilization, gluconeogenesis, lipogenesis and fatty acid oxidation.

MATERIALS AND METHODS

Experimental and sampling procedure

Juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were reared in the INRA experimental facilities at Donzacq (Landes, France) at a constant water temperature of 17.5±0.5°C, under natural photoperiod. They were fed a standard trout commercial diet (T-3P classic, Skretting, Fontaine-les-Vervins, France) during the acclimatization period. Fish (mean ± s.e.m. body mass 140±10 g) were distributed into four tanks per treatment (15 fish per 70 l tank). Prior to the feeding trials, fish were food deprived for 72 h – the time required to ensure the complete emptying of the digestive tract. After this period, trout were sedated with benzocaine (10 mg l^{-1}) and treated with a single i.p. injection of rapamycin (0.5 mg kg^{-1}) or vehicle (75% DMSO and 25% saline solution, NaCl 0.9%) at 100 µl 100 g^{-1} body mass. Two tanks per treatment (vehicle or rapamycin) were re-fed with the commercial diet (T-3P classic) to apparent satiation 3 h after the injection while the other two tanks remained fasted. Four fish per tank (two tanks per condition) were randomly sampled at 2, 4 and 24 h after re-feeding (N=8). Trout were anaesthetized with benzocaine (30 mg l^{-1}) and killed by a sharp blow to the head. Blood was removed from the caudal vein into heparinized syringes and centrifuged (3000 ***g***, 5 min); the recovered plasma was immediately frozen and kept at –20°C. The stomach contents of each fish were checked to confirm that the fish had effectively ingested the diet. Livers were dissected and immediately frozen in liquid nitrogen and kept at –80°C. The experiments were carried out in accordance with the clear boundaries of EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decree no. 2001-464, 29 May 2001). The investigators carrying out the experiment had ‘level 1’ or ‘level 2’ certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002-36, 14 April 2002).

Plasma metabolite analysis

Plasma glucose (Glucose RTU, bioMérieux, Marcy l’Etoile, France), triglycerides (PAP 150, bioMérieux) and free fatty acid (NEFA C kit, Wako Chemicals, Neuss, Germany) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid levels were determined by the ninhydrin reaction (Moore, 1968), with glycine as standard.

Western blot analysis

Frozen livers (N=6; 200 mg) from 2 h re-fed trout were homogenized on ice with an ULTRA-TURRAX homogenizer (IKA-WERKE, Staufen, Germany) in 2 ml of buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA (pH 7.4), 100 mmol l^{-1} NaF, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% Triton X-100, 0.5% NP-40-Igepal and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged at 1500 ***g*** for 15 min at 4°C and supernatant fractions were then centrifuged at 20,000 ***g*** at 4°C for 30 min. The resulting supernatant fractions were recovered and stored at −80°C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (10 µg of total protein for Akt/TOR/S6/4E-BP1 and 20 µg for S6K1/FoxO1) were subjected to SDS-PAGE and western blotting using the appropriate antibody. Anti-phospho-Akt (Ser473) (no. 9271), anti-phospho-Akt (Thr308) (no. 9275), anti-carboxyl terminal Akt (no. 9272), anti-phospho-TOR (Ser2448) (no. 2971), anti-TOR (no. 2972), anti-phospho-S6 protein kinase 1 (Thr389) (no. 9205), anti-S6 protein kinase 1 (no. 9202), anti-phospho-S6 (Ser235/236) (no. 4856), anti-S6 (no. 2217), anti-phospho-4E-BP1 (Thr37/46) (no. 9459), anti-4E-BP1 (no. 9452), anti-phospho-FoxO1 (Thr24)/FoxO3a (Thr32) (no. 9464), anti-phospho-glycogen synthase kinase 3α/β (GSK 3α/β; Ser21/9) (no. 9331), anti-GSK 3α (no. 9338) and anti-β-tubulin (no. 2146) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). Anti-FoxO1 (no. 1874-1) was purchased from Epitomics (Burlingame, CA, USA). All of these antibodies [except anti-phospho-Akt (Thr308), anti-phospho-GSK 157 3α/β (Ser21/9) and anti-GSK 3α] successfully cross-reacted with rainbow trout proteins (Kamalam et al., 2012; Seiliez et al., 2008; Seiliez et al., 2011b). For anti-phospho-Akt (Thr308), anti-phospho-GSK 3α/β (Ser21/9) and anti-GSK 3α antibody, the molecular weight and amino acid sequences were monitored in the SIGENAE database [information system of the Analysis of Breeding Animals’ Genome (AGENAE); http://www.sigenae.org/] to check for a good conservation of the antigen sequence. Membranes were washed then incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (v.3.0, LI-COR Biosciences).

Gene expression analysis: real-time PCR

Liver samples (N=6) for gene expression were collected from fasted fish and 4 and 24 h re-fed fish, the time interval being based on the post-prandial hepatic gene expression peak of rainbow trout (Mennigen et al., 2012). Total RNA was extracted from −80°C frozen livers using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s recommendations, quantified by spectrophotometry (absorbance at 260 nm) and its integrity assessed using agarose gel electrophoresis. A 1 µg sample of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France) according to the manufacturers’ instructions. Target gene expression levels were determined by quantitative real-time (q)RT-PCR, using specific primers (Skiba-Cassy et al., 2009).

qRT-PCR was carried out on a LightCycler 480 II (Roche Diagnostics) using LightCycler 480 SYBR Green I Master (Roche Diagnostics). qRT-PCR analyses were focused on several key enzymes of hepatic metabolism: glucokinase (GK; EC 2.7.1.2) for glucose phosphorylation; 6-phosphofructo-1-kinase (6PF1K; EC 2.7.1.11) and pyruvate kinase (PK; EC 2.7.1.40) for glycolysis; glucose-6-phosphatase (G6Pase; EC 3.1.3.9) for glucose dephosphorylation; fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) and phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) for gluconeogenesis; glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) for pentose phosphate shunt; ATP citrate lyase (ACLY; EC 2.3.3.8), fatty acid synthase (FAS; EC 2.3.1.85) and the transcription factor SREBP1c for lipogenesis; and carnitine palmitoyltransferase 1 (CPT1; EC 2.3.1.21) and 3-hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35) for fatty acid oxidation. When different isoforms of a gene were known in rainbow trout (as for CPT1), gene expression analysis was performed on each isoform. Elongation factor-1alpha (EF1α) was employed as a non-regulated reference gene, as previously used in rainbow trout, and it was stably expressed in our investigations (data not shown).

PCR was performed using 2 µl of the diluted cDNA (76 times diluted) mixed with 0.24 µl of each primer (10 µmol l^{-1}), 3 µl LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.52 µl DNase/RNase/protease-free water (5 prime GmbH, Hamburg, Germany) in a total volume of 6 µl. The PCR protocol was initiated at 95°C for 10 min for initial denaturation of the cDNA and hot-start Taq polymerase activation, followed by 45 cycles of a three-step amplification program (15 s at 95°C; 10 s at melting temperature T_m (59–65°C); 4.8 s at 72°C), according to the primer set used. Melting curves were systematically monitored (5 s at 95°C; 1 min at 65°C; temperature slope at 0.11°C s^{-1} from 65 to 97°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and also negative controls (reverse transcriptase- and RNA-free samples). Relative quantification of target gene expression was determined using the ΔΔCT method by the LightCycler 480 software (v.SW 1.5, Roche Diagnostics). PCR efficiency, which was measured by the slope of a standard curve using serial dilutions of cDNA, ranged between 1.85 and 2.

Enzyme activity analysis

Enzyme activity was measured from liver samples (N=6) collected from 24 h re-fed trout. Liver samples for GK enzyme activity were homogenized in 10 volumes of ice-cold buffer [80 mmol l^{-1} Tris, 5 mmol l^{-1} EDTA, 2 mmol l^{-1} DTT, 1 mmol l^{-1} benzamidine, 1 mmol l^{-1} 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pH 7.6] and centrifuged at 900 ***g*** at 4°C for 10 min. GK activity was assayed immediately in the supernatant as described elsewhere (Figueiredo-Silva et al., 2010). Liver samples for FAS enzyme activity were homogenized in four volumes of ice-cold buffer (0.02 mol l^{-1} Tris-HCl, 0.25 mol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 0.1 mol l^{-1} NaF, 0.5 mmol l^{-1} PMSF, 0.01 mol l^{-1} β-mercaptoethanol, pH 7.4), centrifuged at 24,000 ***g*** at 4°C for 20 min and the supernatant assayed immediately for FAS enzyme activity in pre-established conditions (Figueiredo-Silva et al., 2010). Enzyme activity, defined as micromoles of substrate converted to product per minute, at 37°C, was expressed per gram of liver tissue. All measurements were performed in duplicate.

Statistical analysis

The data for post-prandial plasma metabolites are expressed as means ± s.e.m. (N=8). The results of western blot, gene expression and enzyme activity are expressed as means + s.e.m. (N=6). The effect of re-feeding, i.p. administration of rapamycin or vehicle, and their interaction were analysed using two-way ANOVA (P<0.05). When the interaction was significant, the results were further analysed using one-way ANOVA and a Tukey test. For all statistical analyses, the level of significance was set at P<0.05.

RESULTS

Plasma metabolite levels

Re-feeding significantly increased glucose levels at 4 h (P<0.01) and 24 h (P<0.001) and free amino acid levels at 24 h (P<0.001), while re-feeding markedly decreased free fatty acid at 4 h (P<0.001) (Table 1). There was no significant difference in the effects of vehicle and rapamycin on the plasma glucose, free fatty acid and free amino acid levels (P>0.05).

Akt/TOR signalling pathway

To validate an inhibitory effect on TOR signalling, we investigated the Akt/TOR signalling pathway in rainbow trout livers using western blot analyses. As illustrated in Fig. 1, re-feeding resulted in enhanced phosphorylation of Akt on Ser473 (P<0.001) and Thr308 (P<0.001), FoxO1 on Thr24 or FoxO3a on Thr32 (P=0.02), GSK 3α/β on Ser21/9 (P<0.01), TOR on Ser2448 (P<0.001), S6K1 on Thr389 (P<0.01), S6 on Ser235/236 (P<0.01) and 4E-BP1 on Thr37/46 (P<0.001) proteins. The levels of these total proteins were not modified by feeding or treatment, except for GSK 3α, which was significantly increased by re-feeding (P<0.01, Fig. 1E), whereas β-tubulin levels were not affected by re-feeding or treatment. Activation of Akt on Ser473 (P=0.52, Fig. 1A) and Thr308 (P=0.34,

Table 1. Post-prandial plasma metabolite levels in rainbow trout subjected to intraperitoneal administration of vehicle or rapamycin, 4 and 24 h after re-feeding

Plasma metabolite (mmol l^{-1})	Time (h)	Vehicle		Rapamycin		*P*-value		
		Fasted	Re-fed	Fasted	Re-fed	Feeding	Treatment	F×T
Glucose	4	3.77±0.12	4.44±0.21	3.75±0.13	4.08±0.20	<0.01	0.27	0.32
	24	3.84±0.11	4.92±0.17	3.81±0.30	5.21±0.38	<0.001	0.61	0.53
Free fatty acids	4	0.29±0.03	0.15±0.02	0.27±0.01	0.18±0.01	<0.001	0.75	0.17
	24	0.29±0.01	0.25±0.02	0.24±0.03	0.25±0.04	0.52	0.29	0.34
Free amino acids	4	3.64±0.22	3.77±0.21	3.47±0.15	4.00±0.21	0.11	0.89	0.33
	24	3.51±0.09	4.84±0.23	3.35±0.16	5.23±0.33	<0.001	0.63	0.20

Data are means ± s.e.m. (N=8). At each post-prandial time (row-wise), the effects of re-feeding (F), intraperitoneal (i.p.) administration of vehicle or rapamycin (T) and their interaction were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant.
F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction of F and T.

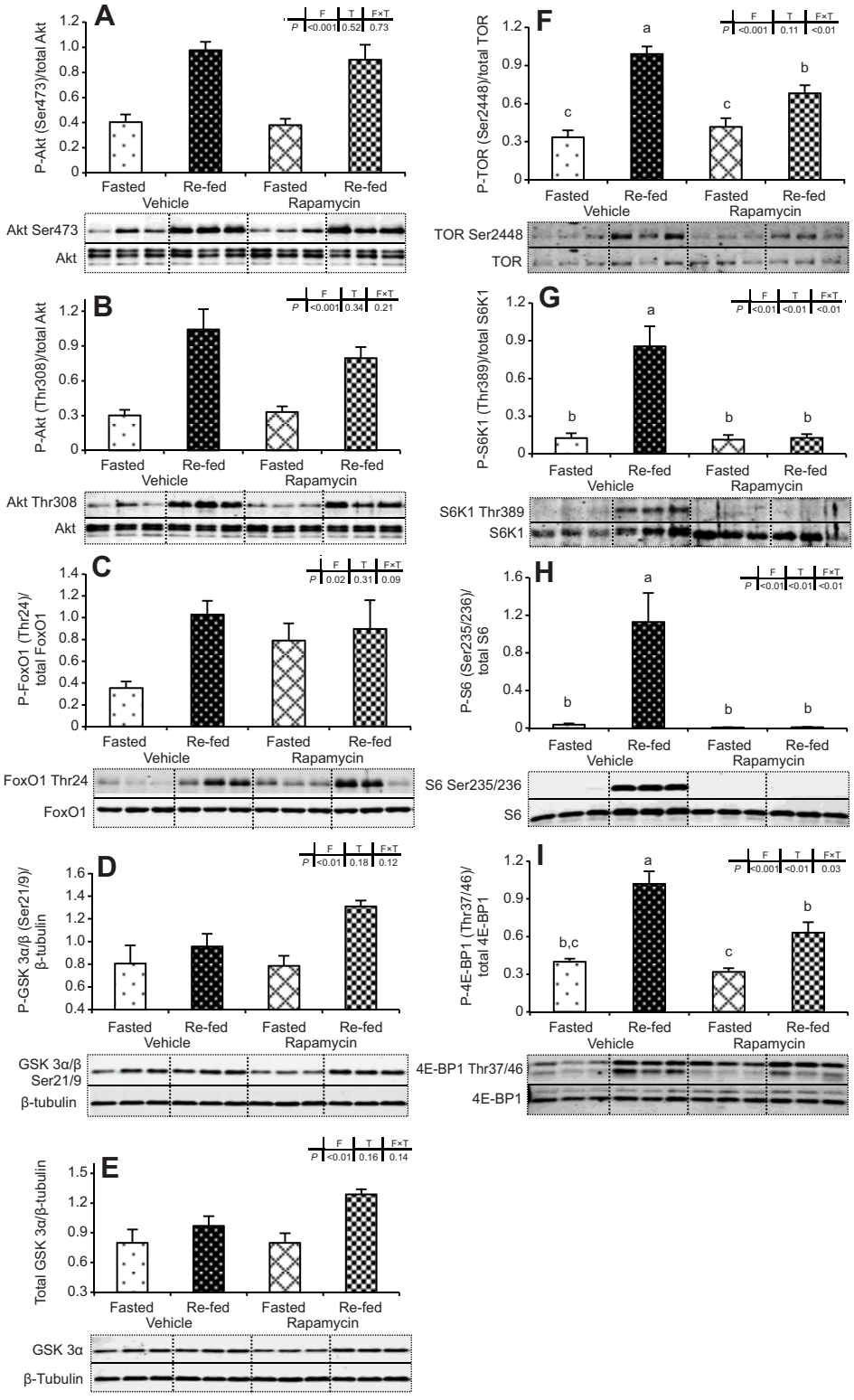

Fig. 1. Western blot analysis of hepatic (A) protein kinase B (Akt; Ser473), (B) Akt (Thr308), (C) forkhead-box class O1 (FoxO1; Thr24), (D,E) glycogen synthase kinase 3α/β (GSK 3), (F) target of rapamycin (TOR), (G) ribosomal protein S6 kinase (S6K1), (H) ribosomal protein S6 (S6) and (I) 4E-binding protein 1 (4E-BP1) protein phosphorylation in rainbow trout subjected to intraperitoneal (i.p.) administration of vehicle or rapamycin, 2 h after re-feeding. Gels were loaded with 10 μg of total protein per lane for Akt/GSK 3/TOR/S6/4E-BP1 and 20 μg for S6K1/FoxO1. A representative blot is shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

Fig. 1B) and the Akt substrates FoxO1 (P=0.31, Fig. 1C) and GSK 3 (P=0.18, Fig. 1D) was not affected by rapamycin, while the phosphorylation of TOR downstream effectors, including S6K1 (P<0.01, Fig. 1G), S6 (P<0.01, Fig. 1H) and 4E-BP1 (P<0.01, Fig. 1I) was totally or partially inhibited by rapamycin. The phosphorylation of S6K1 and S6 in vehicle re-fed fish increased 11- and 31-fold, respectively, compared with vehicle fasted fish, while the phosphorylation of these two proteins was totally abolished in rapamycin-treated fish, irrespective of the feeding status. Re-feeding induced a 2.0-fold increase in 4E-BP1 phosphorylation in rapamycin-treated fish, but this increase was significantly lower compared with the induction observed in vehicle controls. An interaction between feeding and treatment was observed for TOR protein phosphorylation (P<0.01, Fig. 1F), with TOR phosphorylation in vehicle re-fed fish being markedly higher than that in rapamycin re-fed fish.

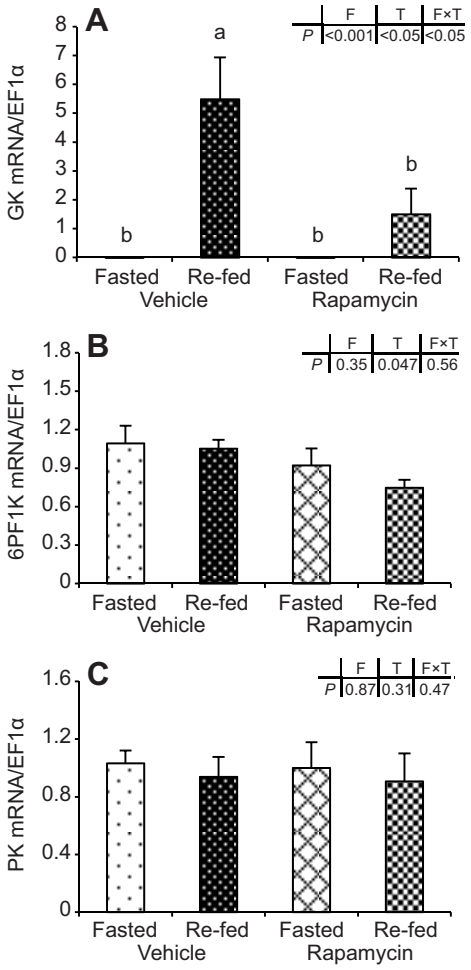

Fig. 2. Gene expression of selected glycolytic enzymes in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin. (A) Glucokinase (GK), (B) 6-phosphofructo-1-kinase (6PF1K) and (C) pyruvate kinase (PK) mRNA levels were measured using quantitative real-time (q)RT-PCR, 24 h after re-feeding. Expression values are normalized with elongation factor-1 alpha (EF1α)-expressed transcripts. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

mRNA levels of target genes

To determine the potential involvement of the TOR signalling pathway in the regulation of hepatic intermediary metabolism-related gene expression, we performed qRT-PCR on genes related to glucose and lipid metabolism. Samples collected at 4 h after re-feeding were unaffected by feeding or treatment (data not shown); the results summarized in Figs 2–5 correspond to samples collected 24 h after re-feeding. The results regarding glycolytic enzyme genes are presented in Fig. 2. GK mRNA levels were upregulated several hundredfold by re-feeding in the vehicle treatment, while this upregulation was significantly suppressed in the rapamycin treatment. Gene expression of 6PF1K was only slightly affected by the treatment (P=0.047, Fig. 2B) and that of PK remained stable irrespective of feeding (P=0.87, Fig. 2C) or treatment (P=0.31, Fig. 2C). As illustrated in Fig. 3A, re-feeding significantly increased (12-fold) FAS gene expression in the vehicle treatment, whereas this induction was totally inhibited by rapamycin. A similar pattern was also observed for SREBP1c mRNA levels (P<0.01, Fig. 3B), with a decrease in rapamycin re-fed fish compared with vehicle re-fed fish. Regardless of feeding or treatment, no significant variation was observed for G6PDH or ACLY mRNA levels. Regarding the mRNA expression of CPT1, both isoforms, CPT1a (Fig. 4A) and CPT1b (Fig. 4B), were positively affected by rapamycin. Nonetheless, re-feeding downregulated the expression of CPT1a in rapamycin-treated fish and that of CPT1b in both vehicle and rapamycin treatments. HOAD mRNA levels were also modulated by the treatment (P<0.01, Fig. 4C), with lower HOAD gene expression in the rapamycin treatment compared with the vehicle treatment irrespective of feeding status. In terms of gluconeogenesis (Fig. 5), mRNA levels of PEPCK (P<0.05), FBPase (P<0.05) and G6Pase (P<0.001) were downregulated by re-feeding irrespective of the treatment.

Enzyme activity analysis

To substantiate the results from gene expression analysis, two hepatic enzyme activities, GK and FAS, were determined 24 h after re-feeding (Fig. 6). Re-feeding enhanced GK enzyme activity 7.2-fold in vehicle-treated fish, but this increase was totally abolished by rapamycin treatment. An interaction between feeding and treatment was recorded for FAS enzyme activity (P<0.05, Fig. 6B), showing that rapamycin significantly decreased FAS enzyme activity in rapamycin re-fed fish compared with vehicle re-fed fish.

DISCUSSION

The regulation of metabolism-related gene expression by the TOR pathway has been investigated *in vitro* using several cellular models including primary culture of rainbow trout hepatocytes (Lansard et al., 2010). *In vivo*, most studies have focused on the chronic effects of rapamycin administration in mammals (Fang et al., 2013; Houde et al., 2010; Lamming et al., 2012; Sarbassov et al., 2006; Yang et al., 2012). In these experiments, modification of intermediary metabolism-related gene expression was not the only target of inhibition. For instance, plasma metabolites (Blum, 2002; Cunningham et al., 2007; Hagiwara et al., 2012), glucose intolerance (Chang et al., 2009; Houde et al., 2010; Lamming et al., 2012), insulin resistance (Blum, 2002; Hagiwara et al., 2012), insulin sensitivity (Wullschleger et al., 2006; Yang et al., 2012), islet mass (Yang et al., 2012) and other metabolic aspects have been modified and consequently influenced the expression of target genes. Here, we performed single i.p. administration of rapamycin to achieve an acute inhibition of the TOR signalling pathway in order to determine the potential direct involvement of this pathway in the regulation of post-prandial hepatic lipid and glucose metabolism-related gene expression in rainbow trout.

Akt/TOR signalling pathway

Investigation of the Akt/TOR signalling pathway revealed that administration of rapamycin significantly inhibits the post-prandial phosphorylation of TOR and its downstream effectors S6K1, S6 and 4E-BP1, without affecting Akt, FoxO1/FoxO3 and GSK 3α/β. These results indicate that the activation of the TOR pathway *in vivo* can be successfully blocked by a single injection of rapamycin, which is consistent with our previous finding in primary cultures of rainbow trout hepatocytes (Lansard et al., 2010) and other investigations in mammals (Owen et al., 2012a; Yang et al., 2012). Loss of function of TORC2 in mice through rictor or mLST8 ablation inhibits the phosphorylation of Akt on Ser473 (Hagiwara et al., 2012; Hresko and Mueckler, 2005), and its substrates FoxO1 and GSK 3α/β, while keeping the phosphorylation of TORC1 and

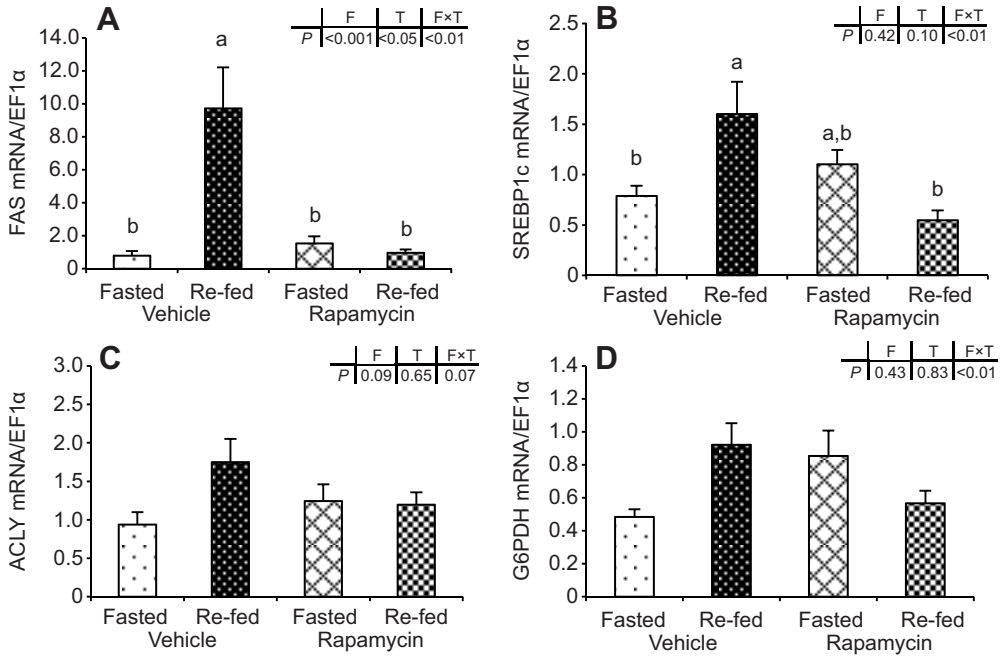

Fig. 3. Gene expression of selected enzymes and transcription factors involved in NADPH generation and lipogenesis in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. (A) Fatty acid synthase (FAS), (B) sterol regulatory element binding protein 1-like (SREBP1c), (C) ATP citrate lyase (ACLY) and (D) glucose 6-phosphate dehydrogenase (G6PDH) mRNA levels were measured using qRT-PCR. Expression values are normalized with EF1α-expressed transcripts. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

its downstream targets intact (Hagiwara et al., 2012; Sarbassov et al., 2005). In the present study, post-prandial phosphorylation of Akt Ser473, FoxO1/FoxO3 and GSK 3α/β was not affected by the administration of rapamycin, suggesting that acute administration of rapamycin in trout only inhibits the phosphorylation of TORC1, without affecting TORC2. Administration of rapamycin in trout reduces but does not totally abolish 4E-BP1 phosphorylation. This lower effect of rapamycin on 4E-BP1 could be explained by a partial recovery of 4E-BP1 phosphorylation during the post-prandial period. Indeed, rapamycin has been shown to differentially affect S6K1 and 4E-BP1 phosphorylation *in vitro* with a transient inhibitory effect on 4E-BP1 phosphorylation *versus* a long-term inhibition of S6K1 (Choo et al., 2008).

Lipid metabolism

The liver is an important site of lipid synthesis and export in fish (Tocher, 2003). We observed that TORC1 inhibition impairs post-prandial induction of both FAS gene expression and activity, suggesting that TORC1 controls the enzyme activity of FAS mainly at the transcriptional level. FAS is a downstream target gene of the transcription factor SREBP1c in mammals (Horton et al., 2002), but this link has not been unequivocally established in fish, despite reports in several studies performed in rainbow trout that FAS gene expression is associated with increased expression of SREBP1c (Lansard et al., 2010; Seiliez et al., 2011a; Skiba-Cassy et al., 2009). Our present findings confirm the relationship between SREBP1c and FAS gene expressions in trout and clearly demonstrate *in vivo* that post-prandial regulation of hepatic SREBP1c and FAS gene expression depends on the activation of TORC1.

In mammals, Akt acts at multiple regulatory steps, in both a TORC1-dependent and -independent manner, to control SREBP1c gene expression. A recent study revealed a TORC1-independent pathway by which Akt negatively regulates the expression of Insig-2a mRNA to promote SREBP1c expression (Yecies et al., 2011). Another potential mechanism involves Akt-mediated phosphorylation and inhibition of GSK 3, which induces the reduction of SREBP1c mRNA levels (Bengoechea-Alonso and Ericsson, 2009; Krycer et al., 2010). The present data indicate that the phosphorylation of Akt and GSK 3 is not modified after acute rapamycin administration, suggesting that the regulation of SREBP1c gene expression and lipogenesis in rainbow trout liver occurs at least in part independently of TORC2 and GSK 3 activation.

SREBP1c gene expression is induced by re-feeding and this induction is inhibited by rapamycin administration, suggesting that activation of TORC1 in trout liver is both necessary and sufficient to stimulate SREBP1c expression and its downstream targets. In mammals, TORC1 regulates SREBP1c mRNA induction, probably through the control of lipin 1 localization (Peterson et al., 2011). Peterson and colleagues (Peterson et al., 2011) demonstrated that TORC1 directly phosphorylates lipin 1, blocking lipin 1 nuclear entry, thereby promoting nuclear remodelling and mediating the effects of TORC1 on the SREBP target gene. Another potential mechanism by which TORC1 mediates SREBP1c maturation is *via* S6K1 (Cornu et al., 2013; Li et al., 2010; Owen et al., 2012b; Wang et al., 2011). Although acute administration of rapamycin in trout significantly impairs TORC1 activation and inhibits re-feeding induction of SREBP1c and FAS gene expression in trout, further explorations are needed to implicate lipin 1 or S6K1 as the crucial links in the TORC1 control of lipogenesis.

CPT1 is considered to be a rate-limiting enzyme of fatty acid β-oxidation whose expression is enhanced in fasted situations then rapidly reduced after re-feeding in mammals, birds and rainbow trout (Ryu et al., 2005; Seiliez et al., 2011a; Skiba-Cassy et al., 2007). The present study indicates that inhibition of CPT1 gene expression after re-feeding in trout is associated with the activation of Akt irrespective of TORC1 inhibition, suggesting that the regulation of β-oxidation might be dependent on Akt activation and at least partly independent of TORC1 phosphorylation. It has been proposed that Akt phosphorylates and inhibits the forkhead family transcription factor FoxA2, thereby controlling lipid export, fatty acid oxidation and accumulation of triglycerides in the liver (Wolfrum et al., 2004; Wolfrum and Stoffel, 2006). Administration of rapamycin significantly enhances CPT1 gene expression in both fasted and re-fed trout, while negatively affecting the expression of HOAD. The ability of rapamycin to affect gene expression has already been established *in vitro* (Deval et al., 2009). Further investigations are

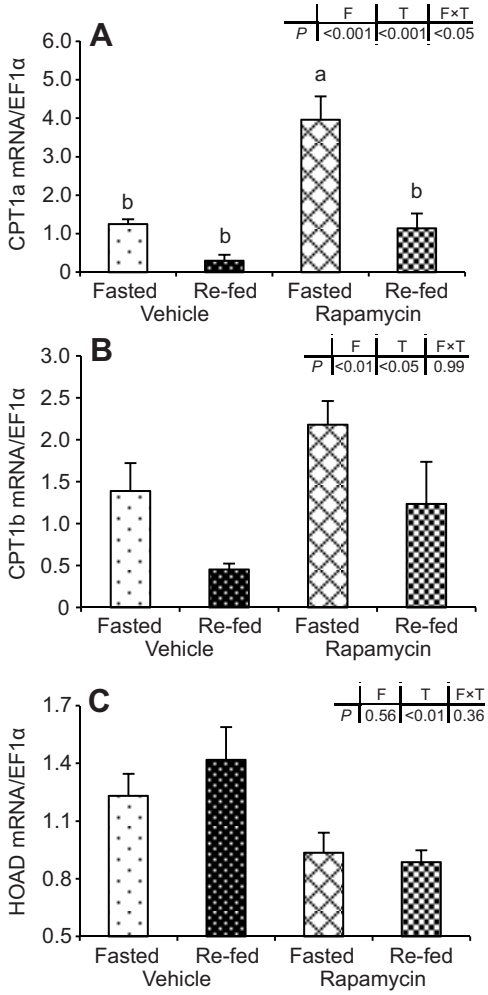

Fig. 4. Gene expression of selected fatty acid oxidation enzymes in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. (A) Carnitine palmitoyl transferase isoforms a (CPT1a) and (B) b (CPT1b) and (C) 3-hydroxyacyl-CoA dehydrogenase (HOAD) mRNA levels were measured using qRT-PCR. Expression values are normalized with EF1α-expressed transcripts. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

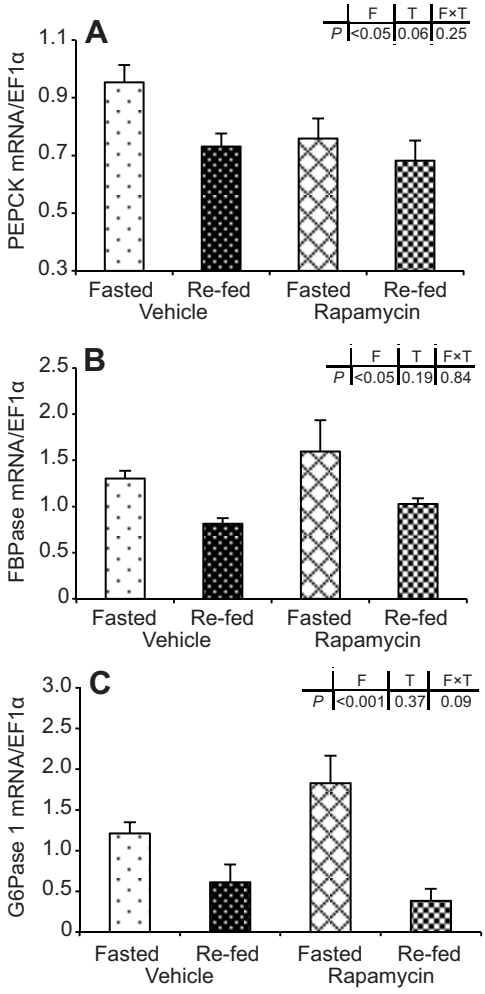

Fig. 5. Gene expression of selected gluconeogenic enzymes in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. (A) Phosphoenolpyruvate carboxykinase (PEPCK), (B) fructose 1,6-bisphosphatase (FBPase) and (C) glucose-6-phosphatase isoform 1 (G6Pase1) mRNA levels were measured using qRT-PCR. Expression values are normalized with EF1α-expressed transcripts. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

needed to determine the TORC1-independent mechanisms by which rapamycin controls the expression of some genes.

Glucose metabolism

GK, the rate-limiting enzyme of glycolysis in the liver, stimulates glycolysis and lipogenesis by enhancing glucose flux, including production of acetyl-CoA for lipid synthesis (Foufelle and Ferré, 2002). Unlike mammals, in which GK gene expression is mainly controlled by insulin (Iynedjian et al., 1989), expression of GK in rainbow trout is highly and predominantly controlled by dietary carbohydrates and glucose (Panserat et al., 2001a; Seiliez et al., 2011a). The present study provides the first evidence that rapamycin inhibits the post-prandial induction of GK enzyme at molecular and activity levels in trout liver, suggesting that GK and FAS activity may be controlled at a transcriptional level in a TORC1-dependent manner. This is consistent with our previous finding in rainbow trout hepatocytes (Lansard et al., 2010) and investigations in mammals (Düvel et al., 2010; Gosmain et al., 2005). How TORC1 regulates GK is quite complex: SREBP1c gene expression is involved in the regulation of lipid metabolism and also controls the expression of glucose metabolism-related genes, such as glucokinase and hexokinase-2 (HKII) in different tissues and cellular models (Ferré and Foufelle, 2007; Foufelle and Ferré, 2002; Gosmain et al., 2005). Other investigations demonstrate that TORC1 increases glycolysis by activating the transcription and translation of HIF1a (Düvel et al., 2010; Majumder et al., 2004), a positive regulator of many glycolytic genes. Furthermore, Düvel and colleagues (Düvel et al., 2010) revealed that HIF1a is stimulated by TORC1-mediated inhibition of 4E-BP1 and phosphorylation of S6K1. Although phosphorylation of 4E-BP1 and S6K1 as well as SREBP1c and GK gene expression are decreased in the liver of rapamycin re-

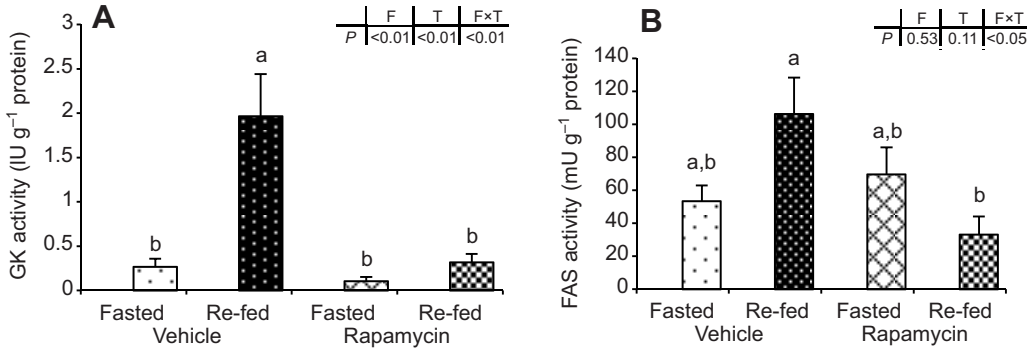

Fig. 6. Enzyme activity of (A) GK and (B) FAS in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. Enzyme activity is defined as micromoles of substrate converted to product, per minute, at 37°C and is expressed per gram of liver tissue. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

fed trout, further investigations are required to determine the molecular mechanisms by which TORC1 regulates GK gene expression.

Despite a significant induction of GK gene expression, expression of 6PF1K and PK is poorly regulated by re-feeding or i.p. rapamycin administration, which is in agreement with a recent study showing that expression of 6PF1K in trout liver, muscle or adipose tissue is not much modified by dietary composition (Kamalam et al., 2012). In mammals, PK gene expression is subject to dietary and hormonal regulation mainly through carbohydrates, insulin and glucagon. Such regulation occurs at the transcriptional level (Hagiwara et al., 2012; Yamada and Noguchi, 1999) and also through post-transcriptional modification of the protein including phosphorylation and dephosphorylation (Assimacopoulos-Jeannet and Jeanrenaud, 1990). In fish, PK activity seems to be mainly controlled by re-feeding through post-translational mechanisms (Panserat et al., 2001b; Skiba-Cassy et al., 2009), explaining the absence of modification of PK gene expression after re-feeding in the present study.

In contrast with our previous observations showing that rapamycin inhibits insulin downregulation of G6Pase expression *in vitro* (Lansard et al., 2010), our present data indicate that rapamycin fails to interfere in the post-prandial downregulation of gluconeogenic gene expression, suggesting a weak contribution of TORC1 in the inhibition of gluconeogenesis in re-fed trout or implicating other endocrine or nutritional factors with counteracting effects. In rats, rapamycin was even able to stimulate G6Pase and PEPCK gene expression (Houde et al., 2010). This discrepancy between trout and rat may be attributed to the length of the rapamycin treatment. Unlike trout that received a single injection of rapamycin and exhibited poor modification of plasma metabolites, rats were submitted to a 2 week chronic rapamycin treatment and presented severe glucose intolerance and insulin resistance associated with impaired β-cell function that could have significantly influenced the expression of G6Pase and PEPCK.

Perspective and significance

The present study demonstrates the functional importance of hepatic TORC1 in the regulation of mRNA levels related to glucose utilization and lipogenesis in rainbow trout. This constitutes the first *in vivo* demonstration of the role of TORC1 in the metabolic response of the liver to nutritional status in fish. Hepatic TORC1 inhibition results in decreased SREBP1c gene expression and suppressed FAS and GK at gene expression and activity levels. Our data support a direct effect of TORC1 on SREBP1c, FAS and GK gene expression inasmuch as fish were subjected to a single injection of rapamycin and did not exhibit drastic changes in post-prandial metabolic parameters. Previous studies have reported that nutritional regulation of the TORC1 signalling pathway and glucose- and lipid-related gene expression are highly influenced by the dietary protein supply in rainbow trout (Seiliez et al., 2011a; Skiba-Cassy et al., 2013). In this carnivorous fish, a strict reduction of dietary protein supply impaired post-prandial activation of the TORC1 pathway and also strongly inhibited the expression of genes related to lipogenesis. Together with these studies, our current findings strongly suggest the TORC1 pathway is crucial for dietary protein to control post-prandial lipogenesis in rainbow trout. Further investigations are needed to confirm the role of TORC1 in the metabolic response of the liver to macronutrients, and identify the mechanisms by which TORC1 regulates SREBP1c expression as well as FAS and GK gene expression and enzyme activity. Understanding how amino acids influence metabolism-related gene expression in various physiological situations or metabolic disorders will be of great influence for limiting the development of metabolic pathologies or optimizing growth and development of livestock animals.

LIST OF ABBREVIATIONS

4E-BP1	4E-binding protein 1
6PF1K	6-phosphofructo-1-kinase
ACLY	ATP citrate lyase
Akt	protein kinase B
CPT1	carnitine palmitoyltransferase 1
EF1α	elongation factor-1 alpha
FAS	fatty acid synthase
FBPase	fructose 1,6-bisphosphatase
FKBP12	12-kDa FK506-binding protein
FoxO1	forkhead-box class O1
G6Pase	glucose 6-phosphatase
G6PDH	glucose 6-phosphate dehydrogenase
GK	glucokinase
GSK 3	glycogen synthase kinase 3α/β
HIF1a	hypoxia inducible factor 1a
HK	hexokinase
HOAD	3-hydroxyacyl-CoA dehydrogenase
i.p.	intraperitoneal
PEPCK	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
S6	ribosomal protein S6
S6K1	ribosomal protein S6 kinase

SREBP1c	sterol regulatory element binding protein 1c
TOR	target of rapamycin
TORC	target of rapamycin complex

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AUTHOR CONTRIBUTIONS

W.D. performed data acquisition, data analysis and wrote the manuscript. K.D. contributed to western blot and plasma amino acid analysis. F.T. participated in i.p. administration of rapamycin and fish rearing. J.M. supplied RT-PCR technical assistance. S.S.-C. and S.P. developed the study design. S.S.-C., S.P. and I.S. contributed to manuscript correction. S.S.-C. is the guarantor of this study and takes full responsibility for the data, analysis, statistical analysis and manuscript submission.

COMPETING INTERESTS

No competing interests declared.

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